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PATENT

ALLERGENIC PROTEINS AND PEPTIDES FROM JAPANESE CEDAR
POLLEN

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Related Applications

This application is a continuation-in-part of USSN 07/730,452 filed July 15, 1991, which is a continuation of USSN 07/729,134 filed July 12, 1991. This application takes priority from PCT/US92/05661 filed July 10, 1992. The information contained in these three applications is incorporated herein by reference.

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Background of the Invention

Genetically predisposed individuals, who make up about 10% of the population, become hypersensitized (allergic) to antigens from a variety of environmental sources to which they are exposed. Those antigens that can induce immediate and/or delayed types of hypersensitivity are known as allergens. (King, T.P., *Adv. Immunol.* 23: 77-105, (1976)). Anaphylaxis or atopy, which includes the symptoms of hay fever, asthma, and hives, is one form of immediate allergy. It can be caused by a variety of atopic allergens, such as products of grasses, trees, weeds, animal dander, insects, food, drugs, and chemicals.

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The antibodies involved in atopic allergy belong primarily to the IgE class of immunoglobulins. IgE binds to mast cells and basophils. Upon combination of a specific allergen with IgE bound to mast cells or basophils, the IgE may be cross-linked on the cell surface, resulting in the physiological effects of IgE-antigen interaction. These physiological effects include the release of, among other substances, histamine, serotonin, heparin, a chemotactic factor for eosinophilic leukocytes and/or the leukotrienes, C4, D4, and E4, which cause prolonged constriction of bronchial smooth muscle cells (Hood, L.E. et al. *Immunology* (2nd

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ed.), The Benjamin/Cumming Publishing Co., Inc. (1984)). These released substances are the mediators which result in allergic symptoms caused by a combination of IgE with a specific allergen. Through them, the effects of an allergen are manifested. Such effects may be systemic or local in nature, depending on the route by which the antigen entered the body and the pattern of deposition of IgE on mast cells or basophils. Local manifestations generally occur on epithelial surfaces at the location at which the allergen entered the body. Systemic effects can include anaphylaxis (anaphylactic shock), which is the result of an IgE-basophil response to circulating (intravascular) antigen.

Japanese cedar (*Sugi; Cryptomeria japonica*) pollinosis is one of the most important allergic diseases in Japan. The number of patients suffering from this disease is on the increase and in some areas, more than 10% of the population are affected. Treatment of Japanese cedar pollinosis by administration of Japanese cedar pollen extract to effect hyposensitization to the allergen has been attempted. Hyposensitization using Japanese cedar pollen extract, however, has drawbacks in that it can elicit anaphylaxis if high doses are used, whereas when low doses are used to avoid anaphylaxis, treatment must be continued for several years to build up a tolerance for the extract.

The major allergen from Japanese cedar pollen has been purified and designated as Sugi basic protein (SBP) or *Cry j I*. This protein is reported to be a basic protein with a molecular weight of 41-50 kDa and a pI of 8.8. There appear to be multiple isoforms of the allergen, apparently due in part to differential glycosylation (Yasueda et al. (1983) *J. Allergy Clin. Immunol.* 71: 77-86; and Taniai et al. (1988) *FEBS Letters* 239: 329-332. The sequence of the first twenty amino acids at the N-terminal end of *Cry j I* and a sixteen amino acid internal sequence have been determined (Taniai *supra*).

A second allergen from Japanese cedar pollen having a molecular weight of about 37 kDa known as *Cry j II* has also been reported (Sakaguchi et al. (1990) *Allergy* 45: 309-312). This allergen was found to have no immunological cross-reactivity with *Cry j I*. Most patients with Japanese cedar pollinosis were found to have IgE antibodies to both *Cry j I* and *Cry j II*, however, sera from some patients reacted with only *Cry j I* or *Cry j II*.

In addition to hyposensitization of Japanese cedar pollinosis patients

with low doses of Japanese cedar pollen extract, U.S. patent 4,939,239 issued July 3, 1990 to Matsuhashi et al. discloses a hyposensitization agent comprising a saccharide covalently linked to a Japanese cedar pollen allergen for hyposensitization of persons sensitive to Japanese cedar pollen. This 5 hyposensitization agent is reported to enhance the production of IgG and IgM antibodies, but reduce production of IgE antibodies which are specific to the allergen and responsible for anaphylaxis and allergy. The allergens used in the hyposensitization agent preferably have an NH₂-terminal amino acid sequence of Asp-Asn-Pro-Ile-Asp-Ser-X-Trp-Arg-Gly-Asp-Ser-Asn-Trp-Ala-Gln-Asn-Arg-Met- 10 Lys-, wherein X is Ser, Cys, Thr, or His (SEQ ID NO: 18). Additionally, Usui et al. (1990) *Int. Arch. Allergy Appl. Immunol.* **91**: 74-79 reported that the ability of a Sugi basic protein (i.e., *Cry j I*)-pullulan conjugate to elicit the Arthus reaction was markedly reduced, about 1,000 times lower than that of native Sugi basic protein and suggested that the Sugi basic protein-pullulan conjugate would be a good candidate 15 for desensitization therapy against cedar pollinosis.

The *Cry j I* allergen found in *Cryptomeria japonica* has also been found to be cross-reactive with allergens in the pollen from other species of trees, including *Cupressus sempervirens*. Panzani et al. (*Annals of Allergy* **57**: 26-30 20 (1986)) reported that cross reactivity was detected between allergens in the pollens of *Cupressus sempervirens* and *Cryptomeria japonica* in skin testing, RAST and RAST inhibition. A 50 kDa allergen isolated from Mountain Cedar (*Juniperus sabinaoides*) has the NH₂-terminal sequence AspAsnProIleAsp (SEQ ID NO: 25) (Gross et al, (1978) *Scand. J. Immunol.* **8**: 437-441) which is the same sequence as the first five amino acids of the NH₂ terminal end of the *Cry j I* allergen. The *Cry j* 25 allergen has also been found to be allergenically cross-reactive with the following species of trees: *Cupressus arizonica*, *Cupressus macrocarpa*, *Juniperus virginiana*, *Juniperus communis*, *Thuya orientalis*, and *Chamaecyparis obtusa*.

Despite the attention Japanese cedar pollinosis allergens have received, definition or characterization of the allergens responsible for its adverse effects on people is far from complete. Current desensitization therapy involves treatment with pollen extract with its attendant risks of anaphylaxis if high doses of pollen extract are administered, or long desensitization times when low doses of pollen extract are administered.

Summary of the Invention

The present invention provides nucleic acid sequences coding for the *Cryptomeria japonica* major pollen allergen *Cry j I* and fragments thereof. The 5 present invention also provides isolated *Cry j I* or at least one fragment thereof produced in a host cell transformed with a nucleic acid sequence coding for *Cry j I* or at least one fragment thereof and fragments of *Cry j I* prepared synthetically. As used herein, a fragment of the nucleic acid sequence coding for the entire amino acid sequence of *Cry j I* refers to a nucleotide sequence having fewer bases than the 10 nucleotide sequence coding for the entire amino acid sequence of *Cry j I* and/or mature *Cry j I*. *Cry j I* and fragments thereof are useful for diagnosing, treating, and preventing Japanese cedar pollinosis.

Peptides within the scope of the invention preferably comprise at least one T cell epitope, and more preferably at least two T cell epitopes of *Cry j I*. The 15 invention further provides peptides comprising at least two regions, each region comprising at least one T cell epitope of a Japanese cedar pollen protein allergen. The invention also provides modified peptides having similar or enhanced therapeutic properties as the corresponding, naturally-occurring allergen or portion thereof, but having reduced side effects, as well as modified peptides having 20 improved properties such as increased solubility and stability. Peptides of the invention are capable of modifying, in a Japanese cedar pollen-sensitive individual to whom they are administered, the allergic response of the individual to a Japanese cedar pollen allergen. Methods of treatment or of diagnosis of sensitivity to Japanese cedar pollen in an individual and therapeutic compositions comprising one or more 25 peptides of the invention are also provided. This invention is more particularly described in the appended claims and is described in its preferred embodiments in the following description.

Brief Description of the Drawings

30 Fig. 1a is a graphic representation of affinity purified *Cry j I* on Superdex 75 (2.6 by 60 cm) equilibrated with 10 mM sodium acetate (pH 5.0) and 0.15 M NaCl;

Fig. 1b shows an SDS-PAGE (12.5%) analysis of the fractions from

the major peak shown in Fig 1a;

Fig. 2 shows a Western blot of isoforms of purified native *Cry j I* proteins separated by SDS-PAGE and probed with mAB CBF2;

Fig. 3 is a graphic representation of allergic sera titration of different purified fractions of purified native *Cry j I* using plasma from a pool of fifteen allergic patients;

Figs. 4a-b show the composite nucleic acid sequence from the two overlapping clones JC 71.6 and pUC19JC91a coding for *Cry j I*. The complete cDNA sequence for *Cry j I* is composed of 1312 nucleotides, including 66 nucleotides of 5' untranslated sequence, an open reading frame starting with the codon for an initiating methionine of 1122 nucleotides, and a 3' untranslated region. Figs. 4a-b also show the deduced amino acid sequence of *Cry j I*;

Fig. 5a is a graphic representation of the results of IgE binding reactivity wherein the coating antigen is soluble pollen extract (SPE) from Japanese cedar pollen;

Fig. 5b is a graphic representation of the results of IgE binding reactivity wherein the coating antigen is purified native *Cry j I*;

Fig. 6 is a graphic representation of the results of a competition ELISA with pooled human plasma (PHP) from 15 patients wherein the coating antigen is soluble pollen extract (SPE) from Japanese cedar pollen;

Fig. 7 is a graphic representation of the results of a competition ELISA using plasma from individual patients (indicated by patient numbers) wherein the coating antigen is soluble pollen extract (SPE) from Japanese cedar pollen and the competing antigen is purified native *Cry j I*;

Fig. 8a is a graphic representation of the results from a direct binding ELISA using plasma from seven individual patients (indicated by patient numbers) wherein the coating antigen is soluble pollen extract (SPE) from Japanese cedar pollen;

Fig. 8b is a graphic representation of the results from a direct binding ELISA using plasma from seven individual patients (indicated by patient numbers) wherein the coating antigen is denatured soluble pollen extract which has been denatured by boiling in the presence of a reducing agent, DTT;

Fig. 9 is a graphic representation of a direct ELISA where the wells

were coated with recombinant *Cry j I* (r*Cry j I*) and IgE binding was assayed on individual patients;

5 Fig. 10a is a graphic representation of the results of a capture ELISA using pooled human plasma from fifteen patients wherein the wells were coated with CBF2 (IgG) mAb, PBS was used as a negative antigen control, and the antigen was purified recombinant *Cry j I*;

10 Fig. 10b is a graphic representation of the results of a capture ELISA using rabbit anti-*Amb aI* and II, wherein the wells were coated with 20 µg/ml CBF2 (IgG), PBS was used as a negative antigen control and the antigen was purified recombinant *Cry j I*;

Fig. 11 is a graphic representation of a histamine release assay performed on one Japanese cedar pollen allergic patient using SPE from Japanese cedar pollen, purified native *Cry j I* and recombinant *Cry j I* as the added antigens; and

15 Fig. 12 is a graphic representation of the results of a T cell proliferation assay using blood from patient #999 wherein the antigen is recombinant *Cry j I* protein, purified native *Cry j I* protein, or recombinant *Amb a 1.1*.

Fig. 13 shows various peptides of desired lengths derived from *Cry j I*.

20 Fig. 14 is a graphic representation depicting responses of T cell lines from seventeen patients primed in vitro with purified native *Cry j I* and analyzed for response to various *Cry j I* peptides by percent of responses (positive) with a S.I of at least two (shown over each bar), the mean stimulation index of positive response for the peptide (shown over each bar in parenthesis) and the positivity index (Y axis).

25 Fig. 15 is a graphic representation of the results of a direct binding assay of IgE to certain *Cry j I* peptides, purified native *Cry j I* and r*Cry j I*.

Detailed Description of the Invention

30 The present invention provides nucleic acid sequences coding for *Cry j I*, the major allergen found in Japanese cedar pollen. The nucleic acid sequence coding for *Cry j I* preferably has the sequence shown in Figs. 4a and 4b (SEQ ID NO: 1). The nucleic acid sequence coding for *Cry j I* shown in Figs. 4a and 4b

(SEQ ID NO: 1) contains a 21 amino acid leader sequence from base 66 through base 128. This leader sequence is cleaved from the mature protein which is encoded by bases 129 through 1187. The deduced amino acid sequence of *Cry j I* is also shown in Figs. 4a and 4b (SEQ ID NO: 2). The nucleic acid sequence of the invention codes for a protein having a predicted molecular weight of 38.5 kDa, with a pI of 7.8, and five potential N-linked glycosylation sites. Utilization of these glycosylation sites will increase the molecular weight and affect the pI of the mature protein. The deduced amino acid sequence for the mature protein encoded by the nucleic acid sequence of the invention is identical with the known NH₂-terminal and internal amino acid sequences reported by Taniai et al., *supra*. The NH₂-terminal end of *Cry j I* reported by Taniai et al., *supra* has the sequence shown in SEQ ID NO: 18. The internal sequence reported by Taniai et al., *supra* has the sequence GluAlaPheAsnValGluAsnGlyAsnAlaThrProGlnLeuThrLys (SEQ ID NO: 19). There are sequence polymorphisms observed in the nucleic acid sequence of the invention. For example, single independent nucleotide substitutions at the codons encoding amino acids 38, 51 and 74 (GGA vs. GAA, GTG vs. GCG, and GGG vs. GAG, respectively) of SEQ ID #1 may result in amino acid polymorphisms (G vs. E, V vs. A, and G vs. E, respectively) at these sites. In addition, a single nucleotide substitution has been detected in one cDNA clone derived from *Cryptomeria japonica* pollen collected in Japan. This substitution in the codon for amino acid 60 (TAT vs. CAT) of SEQ ID #1 may result in an amino acid polymorphism (Y vs. H) at this site. Additional silent nucleotide substitutions have been detected. It is expected that there are additional sequence polymorphisms, and it will be appreciated by one skilled in the art that one or more nucleotides (up to about 1% of the nucleotides) in the nucleic acid sequence coding for *Cry j I* may vary among individual *Cryptomeria japonica* plants due to natural allelic variation. Any and all such nucleotide variations and resulting amino acid polymorphisms are within the scope of the invention. Furthermore, there may be one or more family members of *Cry j I*. Such family members are defined as proteins related in function and amino acid sequence to *Cry j I* but encoded by genes at separate genetic loci.

Fragments of the nucleic acid sequence coding for fragments of *Cry j I* are also within the scope of the invention. Fragments within the scope of the invention include those coding for parts of *Cry j I* which induce an immune response

in mammals, preferably humans, such as stimulation of minimal amounts of IgE; binding of IgE; eliciting the production of IgG and IgM antibodies; or the eliciting of a T cell response such as proliferation and/or lymphokine secretion and/or the induction of T cell anergy. The foregoing fragments of *Cry j I* are referred to herein as antigenic fragments. Fragments within the scope of the invention also include those capable of hybridizing with nucleic acid from other plant species for use in screening protocols to detect allergens that are cross-reactive with *Cry j I*. As used herein, a fragment of the nucleic acid sequence coding for *Cry j I* refers to a nucleotide sequence having fewer bases than the nucleotide sequence coding for the entire amino acid sequence of *Cry j I* and/or mature *Cry j I*. Generally, the nucleic acid sequence coding for the fragment or fragments of *Cry j I* will be selected from the bases coding for the mature protein, however, in some instances it may be desirable to select all or a part of a fragment or fragments from the leader sequence portion of the nucleic acid sequence of the invention. The nucleic acid sequence of the invention may also contain linker sequences, modified restriction endonuclease sites and other sequences useful for cloning, expression or purification of *Cry j I* or fragments thereof.

A nucleic acid sequence coding for *Cry j I* may be obtained from *Cryptomeria japonica* plants. However, Applicants have found that mRNA coding for *Cry j I* could not be obtained from commercially available *Cryptomeria japonica* pollen. This inability to obtain mRNA from the pollen may be due to problems with storage or transportation of commercially available pollen. Applicants have found that fresh pollen and staminate cones are a good source of *Cry j I* mRNA. It may also be possible to obtain the nucleic acid sequence coding for *Cry j I* from genomic DNA. *Cryptomeria japonica* is a well-known species of cedar, and plant material may be obtained from wild, cultivated, or ornamental plants. The nucleic acid sequence coding for *Cry j I* may be obtained using the method disclosed herein or any other suitable techniques for isolation and cloning of genes. The nucleic acid sequence of the invention may be DNA or RNA.

The present invention provides expression vectors and host cells transformed to express the nucleic acid sequences of the invention. A nucleic acid sequence coding for *Cry j I*, or at least one fragment thereof may be expressed in bacterial cells such as *E. coli*, insect cells (baculovirus), yeast, or mammalian cells such as

Chinese hamster ovary cells (CHO). Suitable expression vectors, promoters, enhancers, and other expression control elements may be found in Sambrook et al. *Molecular Cloning: A Laboratory Manual*, second edition, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York (1989). Other suitable expression vectors, promoters, enhancers, and other expression elements are known to those skilled in the art. Expression in mammalian, yeast or insect cells leads to partial or complete glycosylation of the recombinant material and formation of any inter- or intra-chain disulfide bonds. Suitable vectors for expression in yeast include YepSec1 (Baldari et al. (1987) *Embo J.* **6**: 229-234); pMFA (Kurjan and Herskowitz (1982) *Cell* **30**: 933-943); JRY88 (Schultz et al. (1987) *Gene* **54**: 113-123) and pYES2 (Invitrogen Corporation, San Diego, CA). These vectors are freely available. Baculovirus and mammalian expression systems are also available. For example, a baculovirus system is commercially available (PharMingen, San Diego, CA) for expression in insect cells while the pMSG vector is commercially available (Pharmacia, Piscataway, NJ) for expression in mammalian cells.

For expression in *E. coli*, suitable expression vectors include, among others, pTRC (Amann et al. (1988) *Gene* **69**: 301-315); pGEX (Amrad Corp., Melbourne, Australia); pMAL (N.E. Biolabs, Beverly, MA); pRIT5 (Pharmacia, Piscataway, NJ); pET-11d (Novagen, Madison, WI) Jameel et al., (1990) *J. Virol.* **64**:3963-3966; and pSEM (Knapp et al. (1990) *BioTechniques* **8**: 280-281). The use of pTRC, and pET-11d, for example, will lead to the expression of unfused protein. The use of pMAL, pRIT5 pSEM and pGEX will lead to the expression of allergen fused to maltose E binding protein (pMAL), protein A (pRIT5), truncated β -galactosidase (PSEM), or glutathione S-transferase (pGEX). When *Cry j I*, fragment, or fragments thereof is expressed as a fusion protein, it is particularly advantageous to introduce an enzymatic cleavage site at the fusion junction between the carrier protein and *Cry j I* or fragment thereof. *Cry j I* or fragment thereof may then be recovered from the fusion protein through enzymatic cleavage at the enzymatic site and biochemical purification using conventional techniques for purification of proteins and peptides. Suitable enzymatic cleavage sites include those for blood clotting Factor Xa or thrombin for which the appropriate enzymes and protocols for cleavage are commercially available from, for example, Sigma Chemical Company, St. Louis, MO and N.E. Biolabs, Beverly, MA. The different

vectors also have different promoter regions allowing constitutive or inducible expression with, for example, IPTG induction (PRTC, Amann et al., (1988) *supra*; pET-11d, Novagen, Madison, WI) or temperature induction (pRIT5, Pharmacia, Piscataway, NJ). It may also be appropriate to express recombinant *Cry j I* in different *E. coli* hosts that have an altered capacity to degrade recombinantly expressed proteins (e.g. U.S. patent 4,758,512). Alternatively, it may be advantageous to alter the nucleic acid sequence to use codons preferentially utilized by *E. coli*, where such nucleic acid alteration would not affect the amino acid sequence of the expressed protein.

Host cells can be transformed to express the nucleic acid sequences of the invention using conventional techniques such as calcium phosphate or calcium chloride co-precipitation, DEAE-dextran-mediated transfection, or electroporation. Suitable methods for transforming the host cells may be found in Sambrook et al. *supra*, and other laboratory textbooks.

The nucleic acid sequences of the invention may also be synthesized using standard techniques.

The present invention also provides a method of producing isolated Japanese cedar pollen allergen *Cry j I* or at least one fragment thereof comprising the steps of culturing a host cell transformed with a nucleic acid sequence encoding Japanese cedar pollen allergen *Cry j I* or at least one fragment thereof in an appropriate medium to produce a mixture of cells and medium containing said Japanese cedar pollen allergen *Cry j I* or at least one fragment thereof; and purifying the mixture to produce substantially pure Japanese cedar pollen allergen *Cry j I* or at least one fragment thereof. Host cells transformed with an expression vector containing DNA coding for *Cry j I* or at least one fragment thereof are cultured in a suitable medium for the host cell. *Cry j I* protein and peptides can be purified from cell culture medium, host cells, or both using techniques known in the art for purifying peptides and proteins including ion-exchange chromatography, gel filtration chromatography, ultrafiltration, electrophoresis and immunopurification with antibodies specific for *Cry j I* or fragments thereof. The terms isolated and purified are used interchangeably herein and refer to peptides, protein, protein fragments, and nucleic acid sequences substantially free of cellular material or culture medium when produced by recombinant DNA techniques, or chemical

precursors or other chemicals when synthesized chemically.

Another aspect of the invention provides preparations comprising Japanese cedar pollen allergen *Cry j I* or at least one fragment thereof synthesized in a host cell transformed with a nucleic acid sequence encoding all or a portion of Japanese cedar pollen allergen *Cry j I*, or chemically synthesized, and isolated Japanese cedar pollen allergen *Cry j I* protein, or at least one antigenic fragment thereof produced in a host cell transformed with a nucleic acid sequence of the invention, or chemically synthesized. In preferred embodiments of the invention the *Cry j I* protein is produced in a host cell transformed with the nucleic acid sequence coding for at least the mature *Cry j I* protein.

Antigenic fragments of an allergen from Japanese cedar pollen, preferably *Cry j I*, may be obtained, for example, by screening peptides recombinantly produced from the corresponding fragment of the nucleic acid sequence of the invention coding for such peptides or synthesized chemically using techniques known in the art. The allergen may be arbitrarily divided into fragments of a desired length with no overlap of the peptides, or preferably divided into overlapping fragments of a desired length. The fragments are tested to determine their antigenicity (e.g. the ability of the fragment to induce an immune response). If fragments of Japanese cedar pollen allergen, e.g. *Cry j I* are to be used for therapeutic purposes, then the fragments of Japanese cedar pollen allergen which are capable of eliciting a T cell response such as stimulation (i.e., proliferation or lymphokine secretion) and/or are capable of inducing T cell anergy are particularly desirable and fragments of Japanese cedar pollen which have minimal IgE stimulating activity are also desirable. Minimal IgE stimulating activity refers to IgE stimulating activity that is less than the amount of IgE production stimulated by the native *Cry j I* protein. Additionally, for therapeutic purposes, it is preferable to use isolated Japanese cedar pollen allergens, e.g. *Cry j I*, or fragments thereof that do not bind IgE specific for Japanese cedar pollen or bind such IgE to a substantially lesser extent than the purified native Japanese cedar pollen allergen binds such IgE. If the isolated Japanese cedar pollen allergen or fragment or fragments thereof bind IgE, it is preferable that such binding does not result in the release of mediators (e.g. histamines) from mast cells or basophils.

Isolated protein allergens from Japanese cedar pollen or preferred

antigenic fragments thereof, when administered to a Japanese cedar pollen-sensitive individual, or an individual allergic to an allergen cross-reactive with Japanese cedar pollen allergen, such as allergen from the pollen of *Cupressus sempervirens* or *Juniperus sabinaoides* etc. (discussed previously) are capable of modifying the 5 allergic response of the individual to Japanese cedar pollen or such cross-reactive allergen of the individual, and preferably are capable of modifying the B-cell response, T-cell response or both the B-cell and the T-cell response of the individual to the allergen. As used herein, modification of the allergic response of an individual sensitive to a Japanese cedar pollen allergen can be defined as non- 10 responsiveness or diminution in symptoms to the allergen, as determined by standard clinical procedures (See e.g. Varney et al, *British Medical Journal*, 302:265-269 (1990)) including diminution in Japanese cedar pollen induced asthmatic symptoms. As referred to herein, a diminution in symptoms includes any reduction in allergic 15 response of an individual to the allergen after the individual has completed a treatment regimen with a peptide or protein of the invention. This diminution may be subjective (i.e. the patient feels more comfortable in the presence of the allergen). Diminution in symptoms can be determined clinically as well, using standard skin tests as is known in the art.

The isolated *Cry j I* protein or fragments thereof are preferably tested 20 in mammalian models of Japanese cedar pollinosis such as the mouse model disclosed in Tamura et al. (1986) *Microbiol. Immunol.* 30: 883-896, or U.S. patent 4,939,239; or the primate model disclosed in Chiba et al. (1990) *Int. Arch. Allergy Immunol.* 93: 83-88. Initial screening for IgE binding to the protein or fragments thereof may be performed by scratch tests or intradermal skin tests on laboratory 25 animals or human volunteers, or in *in vitro* systems such as RAST (radioallergosorbent test), RAST inhibition, ELISA assay, radioimmunoassay (RIA), or histamine release (see Examples 7 and 8).

Antigenic fragments of the present invention which have T cell stimulating activity, and thus comprise at least one T cell epitope are particularly 30 desirable. T cell epitopes are believed to be involved in initiation and perpetuation of the immune response to a protein allergen which is responsible for the clinical symptoms of allergy. These T cell epitopes are thought to trigger early events at the level of the T helper cell by binding to an appropriate HLA molecule on the surface

of an antigen presenting cell and stimulating the relevant T cell subpopulation. These events lead to T cell proliferation, lymphokine secretion, local inflammatory reactions, recruitment of additional immune cells to the site, and activation of the B cell cascade leading to production of antibodies. One isotype of these antibodies, IgE, is fundamentally important to the development of allergic symptoms and its production is influenced early in the cascade of events, at the level of the T helper cell, by the nature of the lymphokines secreted. A T cell epitope is the basic element or smallest unit of recognition by a T cell receptor, where the epitope comprises amino acids essential to receptor recognition. Amino acid sequences which mimic those of the T cell epitopes and which modify the allergic response to protein allergens are within the scope of this invention.

Exposure of cedar pollen patients to isolated protein allergens of the present invention or to the antigenic fragments of the present invention which comprise at least one T cell epitope and are derived from protein allergens may 5 tolerize or anergize appropriate T cell subpopulations such that they become unresponsive to the protein allergen and do not participate in stimulating an immune response upon such exposure. In addition, administration of the protein allergen of the invention or an antigenic fragment of the present invention which comprises at 10 least one T cell epitope may modify the lymphokine secretion profile as compared with exposure to the naturally-occurring protein allergen or portion thereof (e.g. result in a decrease of IL-4 and/or an increase in IL-2). Furthermore, exposure to 15 such protein allergen or antigenic fragment of such protein allergen may influence T cell subpopulations which normally participate in the response to the allergen such that these T cells are drawn away from the site(s) of normal exposure to the allergen (e.g., nasal mucosa, skin, and lung) towards the site(s) of therapeutic administration 20 of the fragment or protein allergen. This redistribution of T cell subpopulations may 25 ameliorate or reduce the ability of an individual's immune system to stimulate the usual immune response at the site of normal exposure to the allergen, resulting in a diminution in allergic symptoms.

The isolated *Cry j I* protein, and fragments or portions derived therefrom (peptides) can be used in methods of diagnosing, treating and preventing 30 allergic reactions to Japanese cedar pollen allergen or a cross reactive protein allergen. Thus the present invention provides therapeutic compositions comprising

isolated Japanese cedar pollen allergen *Cry j I* or at least one fragment thereof produced in a host cell transformed to express *Cry j I* or at least one fragment thereof, and a pharmaceutically acceptable carrier or diluent. The therapeutic compositions of the invention may also comprise synthetically prepared *Cry j I* or at least one fragment thereof and a pharmaceutically acceptable carrier or diluent.

5 Administration of the therapeutic compositions of the present invention to an individual to be desensitized can be carried out using known techniques. *Cry j I* protein or at least one fragment thereof may be administered to an individual in combination with, for example, an appropriate diluent, a carrier and/or an adjuvant.

10 Pharmaceutically acceptable diluents include saline and aqueous buffer solutions. Pharmaceutically acceptable carriers include polyethylene glycol (Wie et al. (1981) *Int. Arch. Allergy Appl. Immunol.* 64:84-99) and liposomes (Strejan et al. (1984) *J. Neuroimmunol.* 7: 27). For purposes of inducing T cell anergy, the therapeutic composition is preferably administered in nonimmunogenic form, e.g. it does not contain adjuvant. Such compositions will generally be administered by injection (subcutaneous, intravenous, etc.), oral administration, inhalation, transdermal application or rectal administration. The therapeutic compositions of the invention are administered to Japanese cedar pollen-sensitive individuals or individuals sensitive to an allergen which is immunologically cross-reactive with Japanese cedar pollen allergen (i.e. *Cupressus sempervirens*, or *Juniperus sabinaoides*, etc.), at dosages and for lengths of time effective to reduce sensitivity (i.e., reduce the allergic response) of the individual to Japanese cedar pollen. Effective amounts of the therapeutic compositions will vary according to factors such as the degree of sensitivity of the individual to Japanese cedar pollen, the age, sex, and weight of the individual, and the ability of the *Cry j I* protein or fragment thereof to elicit an antigenic response in the individual.

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The *Cry j I* cDNA (or the mRNA from which it was transcribed) or a portion thereof can be used to identify similar sequences in any variety or type of plant and thus, to identify or "pull out" sequences which have sufficient homology to hybridize to the *Cry j I* cDNA or mRNA or portion thereof, for example, DNA from allergens of *Cupressus sempervirens*, *Juniperus sabinaoides* etc., under conditions of low stringency. Those sequences which have sufficient homology (generally greater than 40%) can be selected for further assessment using the method described herein.

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Alternatively, high stringency conditions can be used. In this manner, DNA of the present invention can be used to identify, in other types of plants, preferably related families, genera, or species such as *Juniperus*, or *Cupressus*, sequences encoding polypeptides having amino acid sequences similar to that of Japanese cedar pollen allergen *Cry j I*, and thus to identify allergens in other species. Thus, the present invention includes not only *Cry j I*, but also other allergens encoded by DNA which hybridizes to DNA of the present invention. The invention further includes isolated allergenic proteins or fragments thereof that are immunologically related to *Cry j I* or fragments thereof, such as by antibody cross-reactivity wherein the isolated allergenic proteins or fragments thereof are capable of binding to antibodies specific for the protein and peptides of the invention, or by T cell cross-reactivity wherein the isolated allergenic proteins or fragments thereof are capable of stimulating T cells specific for the protein and peptides of this invention.

Proteins or peptides encoded by the cDNA of the present invention can be used, for example as "purified" allergens. Such purified allergens are useful in the standardization of allergen extracts which are key reagents for the diagnosis and treatment of Japanese cedar pollinosis. Furthermore, by using peptides based on the nucleic acid sequence of *Cry j I*, anti-peptide antisera or monoclonal antibodies can be made using standard methods. These sera or monoclonal antibodies can be used to standardize allergen extracts.

Through use of the peptides and protein of the present invention, preparations of consistent, well-defined composition and biological activity can be made and administered for therapeutic purposes (e.g. to modify the allergic response of a Japanese cedar sensitive individual to pollen of such trees). Administration of such peptides or protein may, for example, modify B-cell response to *Cry j I* allergen, T-cell response to *Cry j I* allergen or both responses. Isolated peptides can also be used to study the mechanism of immunotherapy of *Cryptomeria japonica* allergy and to design modified derivatives or analogues useful in immunotherapy.

Work by others has shown that high doses of allergens generally produce the best results (i.e., best symptom relief). However, many people are unable to tolerate large doses of allergens because of allergic reactions to the allergens. Modification of naturally-occurring allergens can be designed in such a manner that modified peptides or modified allergens which have the same or

enhanced therapeutic properties as the corresponding naturally-occurring allergen but have reduced side effects (especially anaphylactic reactions) can be produced. These can be, for example, a protein or peptide of the present invention (e.g., one having all or a portion of the amino acid sequence of *Cry j I*), or a modified protein or peptide, or protein or peptide analogue. It is possible to modify the structure of a protein or peptide of the invention for such purposes as increasing solubility, enhancing therapeutic or preventive efficacy, or stability (e.g., shelf life *ex vivo*, and resistance to proteolytic degradation *in vivo*). A modified protein or peptide can be produced in which the amino acid sequence has been altered, such as by amino acid substitution, deletion, or addition, to modify immunogenicity and/or reduce allergenicity, or to which a component has been added for the same purpose. For example, the amino acid residues essential to T cell epitope function can be determined using known techniques (e.g., substitution of each residue and determination of the presence or absence of T cell reactivity). Those residues shown to be essential can be modified (e.g., replaced by another amino acid whose presence is shown to enhance T cell reactivity), as can those which are not required for T cell reactivity (e.g., by being replaced by another amino acid whose incorporation enhances T cell reactivity but does not diminish binding to relevant MHC). Another example of a modification of protein or peptides is substitution of cysteine residues preferably with alanine, serine, threonine, leucine or glutamic acid to minimize dimerization via disulfide linkages. Another example of modification of the peptides of the invention is by chemical modification of amino acid side chains or cyclization of the peptide.

In order to enhance stability and/or reactivity, the protein or peptides of the invention can also be modified to incorporate one or more polymorphisms in the amino acid sequence of the protein allergen resulting from natural allelic variation. Additionally, D-amino acids, non-natural amino acids or non-amino acid analogues can be substituted or added to produce a modified protein or peptide within the scope of this invention. Furthermore, proteins or peptides of the present invention can be modified using the polyethylene glycol (PEG) method of A. Sehon and co-workers (Wie et al. *supra*) to produce a protein or peptide conjugated with PEG. In addition, PEG can be added during chemical synthesis of a protein or peptide of the invention. Modifications of proteins or peptides or portions thereof

can also include reduction/ alkylation (Tarr in: *Methods of Protein Microcharacterization*, J.E. Silver ed. Humana Press, Clifton, NJ, pp 155-194 (1986)); acylation (Tarr, *supra*); chemical coupling to an appropriate carrier (Mishell and Shiigi, eds, *Selected Methods in Cellular Immunology*, WH Freeman, San Francisco, CA (1980); U.S. Patent 4,939,239; or mild formalin treatment (Marsh *International Archives of Allergy and Applied Immunology*, 41:199-215 (1971)).

To facilitate purification and potentially increase solubility of proteins or peptides of the invention, it is possible to add reporter group(s) to the peptide backbone. For example, poly-histidine can be added to a peptide to purify the peptide on immobilized metal ion affinity chromatography (Hochuli, E. et al., *BioTechnology*, 6:1321-1325 (1988)). In addition, specific endoprotease cleavage sites can be introduced, if desired, between a reporter group and amino acid sequences of a peptide to facilitate isolation of peptides free of irrelevant sequences. In order to successfully desensitize an individual to a protein antigen, it may be necessary to increase the solubility of a protein or peptide by adding functional groups to the peptide or by not including hydrophobic T cell epitopes or regions containing hydrophobic epitopes in the peptides or hydrophobic regions of the protein or peptide.

To potentially aid proper antigen processing of T cell epitopes within a peptide, canonical protease sensitive sites can be recombinantly or synthetically engineered between regions, each comprising at least one T cell epitope. For example, charged amino acid pairs, such as KK or RR, can be introduced between regions within a peptide during recombinant construction of the peptide. The resulting peptide can be rendered sensitive to cathepsin and/or other trypsin-like enzymes cleavage to generate portions of the peptide containing one or more T cell epitopes. In addition, such charged amino acid residues can result in an increase in solubility of a peptide.

Site-directed mutagenesis of DNA encoding a peptide or protein of the invention (e.g. *Cry j I* or a fragment thereof) can be used to modify the structure of the peptide or protein by methods known in the art. Such methods may, among others, include PCR with degenerate oligonucleotides (Ho et al., *Gene*, 77:51-59 (1989)) or total synthesis of mutated genes (Hostomsky, Z. et al., *Biochem. Biophys. Res. Comm.*, 161:1056-1063 (1989)). To enhance bacterial expression, the

aforementioned methods can be used in conjunction with other procedures to change the eucaryotic codons in DNA constructs encoding protein or peptides of the invention to ones preferentially used in *E. coli*, yeast, mammalian cells, or other eukaryotic cells.

5 Using the structural information now available, it is possible to design *Cry j I* peptides which, when administered to a Japanese cedar pollen sensitive individual in sufficient quantities, will modify the individual's allergic response to Japanese cedar pollen. This can be done, for example, by examining the structure of *Cry j I*, producing peptides (via an expression system, synthetically or otherwise) to be examined for their ability to influence B-cell and/or T-cell responses in Japanese 10 cedar pollen sensitive individuals and selecting appropriate peptides which contain epitopes recognized by the cells. In referring to an epitope, the epitope will be the basic element or smallest unit of recognition by a receptor, particularly immunoglobulins, histocompatibility antigens and T cell receptors where the epitope 15 comprises amino acids essential to receptor recognition. Amino acid sequences which mimic those of the epitopes and which are capable of down regulating allergic response to *Cry j I* can also be used.

It is now also possible to design an agent or a drug capable of blocking or inhibiting the ability of Japanese cedar pollen allergen to induce an 20 allergic reaction in Japanese cedar pollen sensitive individuals. Such agents could be designed, for example, in such a manner that they would bind to relevant anti-*Cry j I* IgEs, thus preventing IgE-allergen binding and subsequent mast cell degranulation. Alternatively, such agents could bind to cellular components of the immune system, resulting in suppression or desensitization of the allergic response to *Cryptomeria* 25 *japonica* pollen allergens. A non-restrictive example of this is the use of appropriate B- and T-cell epitope peptides, or modifications thereof, based on the cDNA/protein structures of the present invention to suppress the allergic response to Japanese cedar pollen. This can be carried out by defining the structures of B- and T-cell epitope peptides which affect B- and T-cell function in *in vitro* studies with blood 30 components from Japanese cedar pollen sensitive individuals.

Protein, peptides or antibodies of the present invention can also be used for detecting and diagnosing Japanese cedar pollinosis. For example, this could be done by combining blood or blood products obtained from an individual to be

assessed for sensitivity to Japanese cedar pollen with an isolated antigenic peptide or peptides of *Cry j I*, or isolated *Cry j I* protein, under conditions appropriate for binding of components in the blood (e.g., antibodies, T-cells, B- cells) with the peptide(s) or protein and determining the extent to which such binding occurs.

5 The present invention also provides a method of producing *Cry j I* or fragment thereof comprising culturing a host cell containing an expression vector which contains a nucleic acid sequence e.g. DNA, encoding all or at least one fragment of *Cry j I* under conditions appropriate for expression of *Cry j I* or at least one fragment. The expressed product is then recovered, using known techniques.
10 Alternatively, *Cry j I* or fragment thereof can be synthesized using known mechanical or chemical techniques.

15 The DNA used in any embodiment of this invention can be cDNA obtained as described herein, or alternatively, can be any oligodeoxynucleotide sequence having all or a portion of a sequence represented herein, or their functional equivalents. Such oligodeoxynucleotide sequences can be produced chemically or enzymatically, using known techniques. A functional equivalent of an oligonucleotide sequence is one which is 1) a sequence capable of hybridizing to a complementary oligonucleotide to which the sequence (or corresponding sequence portions) of SEQ ID NO: 1 or fragments thereof hybridizes, or 2) the sequence (or corresponding sequence portion) complementary to SEQ ID NO: 1, and/or 3) a sequence which encodes a product (e.g., a polypeptide or peptide) having the same functional characteristics of the product encoded by the sequence (or corresponding sequence portion) of SEQ ID NO: 1. Whether a functional equivalent must meet one or both criteria will depend on its use (e.g., if it is to be used only as an oligoprobe, it need meet only the first or second criteria and if it is to be used to produce a *Cry j I* allergen, it need only meet the third criterion).
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30 The present invention also provides isolated peptides derived from Japanese cedar pollen protein. As used herein, a peptide or fragment of a protein refers to an amino acid sequence having fewer amino acid residues than the entire amino acid sequence of the protein from which it is derived. Peptides of the invention include peptides derived from *Cry j I* which comprise at least one T cell epitope of the allergen.

Peptides comprising at least two regions, each region comprising at

least one T cell epitope of Japanese cedar pollen are also within the scope of the invention. Isolated peptides or regions of isolated peptides, each comprising at least two T cell epitopes of a Japanese cedar pollen protein allergen are particularly desirable for increased therapeutic effectiveness. Peptides which are 5 immunologically related (e.g., by antibody or T cell cross-reactivity) to peptides of the present invention are also within the scope of the invention.

Isolated peptides of the invention can be produced by recombinant DNA techniques in a host cell transformed with a nucleic acid having a sequence encoding such peptide as discussed above. The isolated peptides of the invention 10 can also be produced by chemical synthesis. When a peptide is produced by recombinant techniques, host cells transformed with a nucleic acid having a sequence encoding the peptide or the functional equivalent of the nucleic acid sequence are cultured in a medium suitable for the cells and peptides can be purified from cell culture medium, host cells, or both using techniques known in the art for purifying peptides and proteins including ion-exchange chromatography, gel 15 filtration chromatography, ultrafiltration, electrophoresis or immunopurification with antibodies specific for the peptide, the protein allergen Japanese cedar pollen from which the peptide is derived, or a portion thereof. Isolated peptides of the invention are substantially free of cellular material or culture medium when produced by recombinant DNA techniques, or substantially free of chemical precursors or other 20 chemicals when synthesized chemically.

To obtain isolated peptides of the present invention, *Cry j I* is divided into non-overlapping peptides of desired length or overlapping peptides of desired lengths as discussed in Example 6 which can be produced recombinantly, or 25 synthetically. Peptides comprising at least one T cell epitope are capable of eliciting a T cell response, such as T cell proliferation or lymphokine secretion, and/or are capable of inducing T cell anergy (i.e., tolerization). To determine peptides comprising at least one T cell epitope, isolated peptides are tested by, for example, T cell biology techniques, to determine whether the peptides elicit a T cell response or induce T cell anergy. Those peptides found to elicit a T cell response or induce T 30 cell anergy are defined as having T cell stimulating activity.

As discussed in Example 6, human T cell stimulating activity can be tested by culturing T cells obtained from an individual sensitive to Japanese cedar pollen

allergen, (i.e., an individual who has an IgE mediated immune response to Japanese cedar pollen allergen) with a peptide derived from the allergen and determining whether proliferation of T cells occurs in response to the peptide as measured, e.g., by cellular uptake of tritiated thymidine. Stimulation indices for responses by T cells to peptides can be calculated as the maximum CPM in response to a peptide divided by the control CPM. A stimulation index (S.I.) equal to or greater than two times the background level is considered "positive". Positive results are used to calculate the mean stimulation index for each peptide for the group of patients tested. Preferred peptides of this invention comprise at least one T cell epitope and have a mean T cell stimulation index of greater than or equal to 2.0. A peptide having a mean T cell stimulation index of greater than or equal to 2.0 is considered useful as a therapeutic agent. Preferred peptides have a mean T cell stimulation index of at least 2.5, more preferably at least 3.5, more preferably at least 4.0, more preferably at least 5, even more preferably at least 7 and most preferably at least about 9. For example, peptides of the invention having a mean stimulation index of at least 7, as shown in Fig. 14, include ~~CJ1-10, CJ1-16, CJ1-17, CJ1-20, CJ1-22, CJ1-27 and CJ1-32.~~

In addition, preferred peptides have a positivity index (P.I.) of at least about 100, more preferably at least about 250 and most preferably at least about 350. The positivity index for a peptide is determined by multiplying the mean T cell stimulation index by the percent of individuals, in a population of individuals sensitive to Japanese cedar pollen (e.g., preferably at least 15 individuals, more preferably at least 30 individuals or more), who have a T cell stimulation index to such peptide of at least 2.0. Thus, the positivity index represents both the strength of a T cell response to a peptide (S.I.) and the frequency of a T cell response to a peptide in a population of individuals sensitive to Japanese cedar pollen. For example, as shown in Fig. 14, peptide CJ1-22 has a mean S.I. of 18.2 and 64.7% of positive responses in the group of individuals tested resulting in a positivity index of 1177.54. Peptides of *Cry j I* having a positivity index of at least about 100 and a mean T cell stimulation index of at least about 4 include: ~~CJ1-9, CJ1-10, CJ1-16, CJ1-17, CJ1-20, CJ1-22, CJ1-23, CJ1-24, CJ1-27, CJ1-30, CJ1-31, CJ1-32 and CJ1-35.~~

In order to determine precise T cell epitopes by, for example, fine

mapping techniques, a peptide having T cell stimulating activity and thus comprising at least one T cell epitope as determined by T cell biology techniques is modified by addition or deletion of amino acid residues at either the amino or carboxy terminus of the peptide and tested to determine a change in T cell reactivity to the modified peptide. If two or more peptides which share an area of overlap in the native protein sequence are found to have human T cell stimulating activity, as determined by T cell biology techniques, additional peptides can be produced comprising all or a portion of such peptides and these additional peptides can be tested by a similar procedure. Following this technique, peptides are selected and produced

5 recombinantly or synthetically. Peptides are selected based on various factors, including the strength of the T cell response to the peptide (e.g., stimulation index), the frequency of the T cell response to the peptide in a population of individuals sensitive to Japanese cedar pollen, and the potential cross-reactivity of the peptide with other allergens from other species of trees as discussed earlier (e.g. *Cupressus* 10 *Sempervirens*, *Cupressus arizonica*, etc.) or ragweed (*Amb a I.1*). The physical and chemical properties of these selected peptides (e.g., solubility, stability) are examined to determine whether the peptides are suitable for use in therapeutic compositions or whether the peptides require modification as described herein. The ability of the selected peptides or selected modified peptides to stimulate human T 15 cells (e.g., induce proliferation, lymphokine secretion) is determined.

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Additionally, preferred peptides of the invention do not bind immunoglobulin E (IgE) or bind IgE to a substantially lesser extent than the protein allergen from which the peptide is derived binds IgE. The major complications of standard immunotherapy are IgE-mediated responses such as anaphylaxis.

25 Immunoglobulin E is a mediator of anaphylactic reactions which result from the binding and cross-linking of antigen to IgE on mast cells or basophils and the release of mediators (e.g., histamine, serotonin, eosinophil chemotactic factors). Thus, anaphylaxis in a substantial percentage of a population of individuals sensitive to *Cry j I* could be avoided by the use in immunotherapy of a peptide or peptides 30 which do not bind IgE in a substantial percentage (e.g., at least about 75%) of a population of individuals sensitive to *Cry j I* allergen, or if the peptide binds IgE, such binding does not result in the release of mediators from mast cells or basophils. The risk of anaphylaxis could be reduced by the use in immunotherapy of a peptide

or peptides which have reduced IgE binding. Moreover, peptides which have minimal IgE stimulating activity are desirable for therapeutic effectiveness. Minimal IgE stimulating activity refers to IgE production that is less than the amount of IgE production and/or IL-4 production stimulated by the native *Cry j I* protein allergen.

A peptide of the invention, when administered to a Japanese cedar pollen-sensitive individual, is capable of modifying the allergic response of the individual to the allergen. Particularly, peptides of the invention comprising at least one T cell epitope of *Cry j I* or at least two regions derived from *Cry j I*, each comprising at least one T cell epitope, when administered to an individual sensitive to Japanese cedar pollen are capable of modifying T cell response of the individual to the allergen.

A preferred isolated peptide of the invention comprises at least one T cell epitope of the Japanese cedar pollen allergen, *Cry j I* and accordingly the peptide comprises at least approximately seven amino acid residues. For purposes of therapeutic effectiveness, preferred therapeutic compositions of the invention preferably comprise at least two T cell epitopes of *Cry j I*, and accordingly, the peptide comprises at least approximately eight amino acid residues and preferably fifteen amino acid residues. Additionally, therapeutic compositions comprising preferred isolated peptides of the invention preferably comprise a sufficient percentage of the T cell epitopes of the entire protein allergen such that a therapeutic regimen of administration of the composition to an individual sensitive to Japanese cedar pollen, results in T cells of the individual being tolerized to the protein allergen. Synthetically produced peptides of the invention comprising up to approximately forty-five amino acid residues in length, and most preferably up to approximately thirty amino acid residues in length are particularly desirable as increases in length may result in difficulty in peptide synthesis. Peptides of the invention may also be produced recombinantly as described above, and it is preferable that peptides of 45 amino acids or longer be produced recombinantly.

Preferred peptides comprise all or a portion of the areas of major T cell reactivity within the *Cry j I* protein allergen designated herein as, Region 1, Region 2, Region 3, Region 4 and Region 5. Each major area of T cell activity is defined as follows and is shown in Fig. 4 a-b. Region 1 comprises amino acid residues 1-50 of

(SEQ ID NO: 61) (SEQ ID NO: 62)
Cry j I; Region 2 comprises amino acid residues 61-120 of *Cry j I*; Region 3 comprises amino acid residues 131-180 of *Cry j I*; Region 4 comprises amino acid residues 191-280 of *Cry j I*; Region 5 comprises amino acid residues 291-353 of the *Cry j I*. Preferred areas of major T cell reactivity within each Region as shown in Fig. 4 a-b and comprise: amino acid residues 1-40; amino acid residues 81-110; amino acid residues 151-180; amino acid residues 191-240; and amino acid residues 291-330.

Preferred peptides derived from the *Cry j I* protein allergen comprise all or a portion of the following peptides: CJ1-1, CJ1-2, CJ1-3, CJ1-4, CJ1-7, CJ1-8, CJ1-9, CJ1-10, CJ1-11, CJ1-12, CJ1-14, CJ1-15, CJ1-16, CJ1-17, CJ1-18, CJ1-19, CJ1-20, CJ1-21, CJ1-22, CJ1-23, CJ1-24, CJ1-25, CJ1-26, CJ1-27, CJ1-28, CJ1-30, CJ1-31, CJ1-32, CJ1-33, CJ1-34 and CJ1-35 wherein the portion of the peptide preferably has a mean T cell stimulation index equivalent to, or greater than the mean T cell stimulation index of the peptide from which it is derived as shown in Fig. 14. Preferred peptides derived from the *Cry j I* protein allergen more preferable comprise all or a portion of the following peptides: CJ1-9, CJ1-10, CJ1-16, CJ1-17, CJ1-20, CJ1-22, CJ1-23, CJ1-24, CJ1-27, CJ1-30, CJ1-31, CJ1-32 and CJ1-35. Additionally, another preferred antigenic peptide of the invention may comprise more than one Region, i.e., all or a portion of amino acids 151-352 of the amino acid sequence of *Cry j I*, as shown in Fig. 4a-b.

One embodiment of the present invention features a peptide or portion thereof of *Cry j I* which comprises at least one T cell epitope of the protein allergen and has a formula X_n-Y-Z_m . According to the formula, Y is an amino acid sequence selected from the group consisting of CJ1-1, CJ1-2, CJ1-3, CJ1-4, CJ1-7, CJ1-8, CJ1-9, CJ1-10, CJ1-11, CJ1-12, CJ1-14, CJ1-15, CJ1-16, CJ1-17, CJ1-18, CJ1-19, CJ1-20, CJ1-21, CJ1-22, CJ1-23, CJ1-24, CJ1-25, CJ1-26, CJ1-27, CJ1-28, CJ1-30, CJ1-31, CJ1-32, CJ1-33, CJ1-34 and CJ1-35, and preferably selected from the group consisting of CJ1-9, CJ1-10, CJ1-16, CJ1-17, CJ1-20, CJ1-22, CJ1-23, CJ1-24, CJ1-27, CJ1-30, CJ1-31, CJ1-32 and CJ1-35. In addition, X_n are amino acid residues contiguous to the amino terminus of Y in the amino acid sequence of the protein allergen and Z_m are amino acid residues contiguous to the carboxy terminus of Y in the amino acid sequence of the protein allergen. In the formula, n is 0-30 and m is 0-30. Preferably, the peptide or portion thereof has a mean T cell

stimulation index equivalent to greater than the mean T cell stimulation index of Y as shown in Fig. 14.

Another embodiment of the present invention provides peptides comprising at least two regions, each region comprising at least one T cell epitope of *Cry j I* and accordingly each region comprises at least approximately seven amino acid residues. These peptides comprising at least two regions can comprise as many amino acid residues as desired and preferably comprise at least about 14, even more preferably about 30, and most preferably at least about 40 amino acid residues of a *Cry j I* allergen. If desired, the amino acid sequences of the regions can be produced and joined by a linker to increase sensitivity to processing by antigen-presenting cells. Such linker can be any non-epitope amino acid sequence or other appropriate linking or joining agent. To obtain preferred peptides comprising at least two regions, each comprising at least one T cell epitope, the regions are arranged in a configuration different from a naturally-occurring configuration of the regions in the allergen. For example, the regions containing T cell epitope(s) can be arranged in a noncontiguous configuration and can preferably be derived from the same protein allergen. Noncontiguous is defined as an arrangement of regions containing T cell epitope(s) which is different than that of an amino acid sequence present in the protein allergen from which the regions are derived. Furthermore, the noncontiguous regions containing T cell epitopes can be arranged in a nonsequential order (e.g., in an order different from the order of the amino acids of the native protein allergen from which the region containing T cell epitope(s) are derived in which amino acids are arranged from an amino terminus to a carboxy terminus). A peptide can comprise at least 15%, at least 30%, at least 50% or up to 100% of the T cell epitopes of *Cry j I*.

The individual peptide regions can be produced and tested to determine which regions bind immunoglobulin E specific for *Cry j I* and which of such regions would cause the release of mediators (e.g., histamine) from mast cells or basophils. Those peptide regions found to bind immunoglobulin E and cause the release of mediators from mast cells or basophils in greater than approximately 10-15% of the allergic sera tested are preferably not included in the peptide regions arranged to form preferred peptides of the invention.

Additionally, regions of a peptide of the invention preferably

comprise all or a portion of the above discussed preferred areas of major T cell reactivity within *Cry j I* (i.e., Regions 1-5) or the above discussed preferred areas of major T cell activity within each Region (i.e. amino acids from residues 1-40, 81-110, 151-180, 191-240 and 291-330). For example, one region can comprise all or a portion of Region 1 (amino acid residues 1-51) and one region can comprise all or a portion of Region 2 (amino acid residues 61-120). Peptides of the invention can comprise all or a portion of two or more of these Regions (i.e., Regions 1-5) and preferred resulting peptides do not bind IgE and cause the release of mediators from most cells or basophils. Preferred peptides derived from *Cry j I* comprise all or a portion of Region 3, Region 4 and Region 5, and, optionally, Region 1 or Region 2. Further, if one of these Regions is found to bind IgE and cause the release of mediators from mast cells or basophils, then it is preferred that the peptide not comprise such Region, but rather comprises various regions derived from such Region which do not bind IgE or cause release of mediators from mast cells or basophils.

Examples of preferred regions include: ~~CJ1-1, CJ1-2, CJ1-3, CJ1-4, CJ1-7, CJ1-8, CJ1-9, CJ1-10, CJ1-11, CJ1-12, CJ1-14, CJ1-15, CJ1-16, CJ1-17, CJ1-18, CJ1-19, CJ1-20, CJ1-21, CJ1-22, CJ1-23, CJ1-24, CJ1-25, CJ1-26, CJ1-27, CJ1-28, CJ1-30, CJ1-31, CJ1-32, CJ1-33, CJ1-34 AND CJ1-35~~, the amino acid sequences of such regions being shown in Fig. 13, or portions of said regions comprising at least one T cell epitope.

Preferred peptides comprise various combinations of two or more regions, each region comprising all or a portion of the above-discussed preferred areas of major T cell reactivity. Preferred peptides comprise a combination of two or more regions (each region having an amino acid sequence as shown in Fig. 13), including:

~~CJ1-1, CJ1-2 and CJ1-3;~~
~~CJ1-1 and CJ1-2;~~
~~CJ1-9 and CJ1-10;~~
~~CJ1-14, CJ1-15, CJ1-16 and CJ1-17;~~
~~CJ1-20, CJ1-21, CJ1-22, CJ1-23;~~
~~CJ1-20, CJ1-22 and CJ1-23;~~
~~CJ1-22 and CJ1-23;~~

5 CJ1-22, CJ1-23 and CJ1-24;
~~CJ1-30, CJ1-31 and CJ1-32;~~
~~CJ1-31 and CJ1-32;~~
~~CJ1-22, CJ1-23, CJ1-16 and CJ1-17;~~
10 CJ1-22, CJ1-23, CJ1-31 and CJ1-32;
~~CJ1-16, CJ1-17, CJ1-31 and CJ1-32;~~
~~CJ1-9, CJ1-10 and CJ1-16;~~
~~CJ1-17, CJ1-22 and CJ1-23;~~
~~CJ1-16, CJ1-17 and CJ1-20;~~
15 CJ1-31, CJ1-32 and CJ1-20;
~~CJ1-22, CJ1-23, CJ1-1, CJ1-2 and CJ1-3;~~
~~CJ1-16, CJ1-17, CJ1-22 and CJ1-23, CJ1-31 and CJ1-32;~~
~~CJ1-9, CJ1-10, CJ1-16, CJ1-17, CJ1-22 and CJ1-23;~~
~~CJ1-9, CJ1-10, CJ1-16, CJ1-17, CJ1-31 and CJ1-32;~~
20 CJ1-9, CJ1-10, CJ1-22, CJ1-23, CJ1-31 and CJ1-32;
~~CJ1-9, CJ1-10, CJ1-16, CJ1-17, CJ1-22, CJ1-23, CJ1-31 and CJ1-32;~~
~~CJ1-1, CJ1-2, CJ1-16, CJ1-17, CJ1-22 and CJ1-23.~~

25 Isolated *Cry j I* protein or peptides of *Cry j I* within the scope of the invention can be used in methods of treating and preventing allergic reactions to Japanese cedar pollen. Thus, one aspect of the present invention provides therapeutic compositions comprising a peptide of *Cry j I* including at least one T cell epitope, or preferably at least two T cell epitopes, and a pharmaceutically acceptable carrier or diluent. In another aspect, the therapeutic composition comprises a pharmaceutically acceptable carrier or diluent and a peptide comprising at least two regions, each region comprising at least one T cell epitope of *Cry j I*.

30 Preferred therapeutic compositions comprise at least one peptide of *Cry j I*. The composition comprises a sufficient percentage of the T cell epitopes of *Cry j I* such that a therapeutic regimen of administration of the composition to an individual sensitive to Japanese cedar pollen allergen, results in T cells of the individual being tolerized to the protein allergen. More preferably, the composition comprises a sufficient percentage of the T cell epitopes such that at least about 40%, and more preferably at least about 60% of the T cell reactivity of *Cry j I* is included in the

composition. Such compositions can be administered to an individual to treat or prevent sensitivity to Japanese cedar pollen or to an allergen which is immunologically cross-reactive with Japanese cedar pollen allergen.

In yet another aspect of the present invention, a composition is provided comprising at least two peptides (e.g., a physical mixture of at least two peptides), each comprising at least one T cell epitope of *Cry j I*. Such compositions can be administered in the form of a therapeutic composition with a pharmaceutically acceptable carrier or diluent. A therapeutically effective amount of one or more of such compositions can be administered simultaneously or sequentially to an individual sensitive to Japanese cedar pollen.

Preferred compositions and preferred combinations of peptides which can be administered simultaneously or sequentially (comprising peptides having amino acid sequences shown in Fig. 13) include the following combinations:

CJ1-1, CJ1-2 and CJ1-3;

CJ1-1 and CJ1-2;

CJ1-9 and CJ1-10;

CJ1-14, CJ1-15, CJ1-16 and CJ1-17;

CJ1-20, CJ1-21, CJ1-22 and CJ1-23;

CJ1-20, CJ1-22 and CJ1-23;

CJ1-22 and CJ1-23;

CJ1-22, CJ1-23 and CJ1-24;

CJ1-30, CJ1-31 and CJ1-32;

CJ1-31 and CJ1-32;

CJ1-22, CJ1-23, CJ1-16 and CJ1-17;

CJ1-22, CJ1-23, CJ1-31 and CJ1-32;

CJ1-16, CJ1-17, CJ1-31 and CJ1-32;

CJ1-9, CJ1-10 and CJ1-16;

CJ1-17, CJ1-22 and CJ1-23;

CJ1-16, CJ1-17 and CJ1-20;

CJ1-31, CJ1-32 and CJ1-20;

CJ1-22, CJ1-23, CJ1-1, CJ1-2 and CJ1-3;

CJ1-16, CJ1-17, CJ1-22, CJ1-23, CJ1-31 and CJ1-32;

CJ1-9, CJ1-10, CJ1-16, CJ1-17, CJ1-22 and CJ1-23;

B
~~CJ1-9, CJ1-10, CJ1-16, CJ1-17, CJ1-31 and CJ1-32;~~
~~CJ1-9, CJ1-10, CJ1-22, CJ1-23, CJ1-31 and CJ1-32;~~
~~CJ1-9, CJ1-10, CJ1-16, CJ1-17, CJ1-22, CJ1-23, CJ1-31 and CJ1-32;~~
~~CJ1-1, CJ1-2, CJ1-16, CJ1-17, CJ1-22 and CJ1-23.~~

5

The invention is further illustrated by the following non-limiting examples.

Example 1

10 **Purification of Native Japanese Cedar Pollen Allergen (*Cry j I*)**

The following is a description of the work done to biochemically purify the major allergen, *Cry j I* in the native form. The purification was modified from published procedures (Yasueda et al., *J. Allergy Clin. Immunol.* 71:77, 1983).

15 100g of Japanese cedar pollen obtained from Japan (Hollister-Stier, Spokane, WA) was defatted in 1 L diethyl ether three times, the pollen was collected after filtration and the ether was dried off in a vacuum.

20 The defatted pollen was extracted at 4°C overnight in 2 L extraction buffer containing 50 mM tris-HCL, pH 7.8, 0.2 M NaCl and protease inhibitors in final concentrations: soybean trypsin inhibitor (2 µg/ml), leupeptin (1 µg/ml), pepstatin A (1 µg/ml) and phenyl methyl sulfonyl fluoride (0.17 mg/ml). The insoluble material was reextracted with 1.2 L extraction buffer at 4°C overnight and both extracts were combined together and depigmented by batch absorption with Whatman DE-52 DEAE cellulose (200 g dry weight) equilibrated with the extraction buffer.

25 The depigmented material was then fractionated by ammonium sulfate precipitation at 80% saturation (4°C), which removed much of the lower molecular weight material. The resultant partially purified *Cry j I* was either dialyzed in PBS buffer and used in T cell studies (see Example 6) or subjected to further purification (biochemically or by monoclonal affinity chromatography) as described below.

30 The enriched *Cry j I* material was then dialyzed against 50 mM Na-acetate, pH 5.0 at 4°C with 50 mM Na-acetate, pH 5.0 with protease inhibitors. The unbound material (basic proteins) was then applied to a 50 ml cation exchange column (Whatman

CM-52) which was equilibrated at 4°C with 10 mM Na-acetate, pH 5.0 with protease inhibitors. *Cry j I* was eluted in the early fractions of a linear gradient 0.3 M NaCl. The enriched *Cry j I* material was lyophilized and was then purified by FPLC over a 300 ml Superdex 75 column (Pharmacia) at a flow rate of 30 ml/h in 10 mM Na-acetate, pH 5.0 at 25°C.

The purified *Cry j I* was further applied to FPLC S-Sepharose 16/10 column chromatography (Pharmacia) with a linear gradient of 0 - 1 M NaCl at 25°C. *Cry j I*, eluted as the major peak, was subjected to a second gel filtration chromatography. FPLC Superdex 75 column (2.6 by 60 cm)(Pharmacia, Piscataway, NJ) was eluted with a downward flow of 10 mM Na-acetate, pH 5.0 with 0.15 M NaCl at a flow rate of 30 ml/h at 25°C. Fig. 1a shows the chromatography on gel filtration. Only *Cry j I* was detected (Fig. 1b, lane 2 to lane 8). *Cry j I* was fractionated into 3 bands as analyzed by SDS-PAGE using silver staining (Fig. 1b) As shown in Fig. 1b, SDS PAGE (12.5%) analysis of the fractions from the major peak shown in Fig. 1a was performed under reducing conditions. The gel was silver stained using the silver staining kit from Bio-Rad. The samples in each lane were as follows: Lane 1, prestained standard proteins (Gibco BRL) including ovalbumin (43,000 kD), carbonic anhydrase (29,000 kD), and α -lactoglobulin (18,400 kD); lane 2, fraction 36 ; lane 3 fraction 37; lane 4 fraction 38; lane 5 fraction 39 ; lane 6 fraction 41, lane 7 fraction 43; and lane 8 fraction 44. All fractions are shown in Fig. 1a.

These proteins were also analyzed by Western blotting using mouse monoclonal antibody CBF2 (Fig. 2). As shown in Fig. 2, an aliquot of fraction 36 (lane 1), fraction 39, (lane 2) and fraction 43 (lane 3) purified from the Superdex 75 as shown in Fig. 1 was separated by SDS-PAGE, electroblotted onto nitrocellulose and probed with mAB CBF2. Biotinylated goat anti-mouse Ig was used for the second antibody and bound antibody was revealed by 125 I-streptavidin. The monoclonal CBF2 was raised against ragweed allergen *Amb a I* by Dr. D. Klapper (Chapel Hill, NC). Because of the homology between the *Amb a I* and *Cry j I* sequences, a number of antibodies raised against *Amb a I* were tested for reactivity with *Cry j I*. The results showed that CBF2 recognized denatured *Cry j I* as detected by ELISA and Western blotting. In addition, Western blotting also demonstrated that no other bands were detected by CBF2, other than *Cry j I* in the expected molecular weight range (Fig. 2). These results were consistent with the findings from protein sequencing. When fraction 44 and

fraction 39 (Fig 1b) were subjected to N-terminal sequencing, only *Cry j I* sequence was detected.

In summary, three *Cry j I* isoforms of different molecular weight were purified from pollen extract. The molecular weights estimated by SDS-PAGE ranged from 40-35 kD under both reducing and non-reducing conditions. The isoelectric point of these isoforms is approximately 9.5-8.6, with an average pl of 9.0. The N-terminal 20 amino acid sequence was the same in these 3 bands and was identical to previously published *Cry j I* sequence (Taniai et al, *supra*). The 3 isoforms are all recognized by monoclonal antibody CBF2 as shown in the allergic sera titration of different purified subfractions of *Cry j I* using a pool of fifteen allergic patient plasma. They all bind allergic patient IgE (Fig. 3). The difference in molecular weight and isoelectric point in these isoforms might in part be due to post-translational modification, e.g. glycosylation, phosphorylation or lipid content. The possibility that these different isoforms might be due to protease degradation cannot be ruled out at present even though it is unlikely due to the fact that four different protease inhibitors were used during extraction and purification. The other possibility could be due to polymorphism in the gene or alternate splicing in the mRNA though only one major form of *Cry j I* protein has been detected in cDNA cloning studies (see Example 4).

Another approach which may be used to purify native *Cry j I* or recombinant *Cry j I* is immunoaffinity chromatography. This technique provides a very selective protein purification due to the specificity of the interaction between monoclonal antibodies and antigen. For the purpose of producing *Cry j I*-reactive monoclonal antibodies, female Balbl/c mice were obtained from Jackson Labs. Each mouse was initially immunized intraperitoneally with 70-100 µg purified native *Cry j I*, (>99% purity lower band, as shown in Fig. 1b), emulsified in Freund's complete adjuvant. One further intravenous injection of 10 µg purified native *Cry j I* in PBS was given 54 days after the initial injection. The spleen was removed 3 days later and myeloma fusion was conducted as described (Current Protocols in Immunology, 1991, Coligan et al, eds.) using the myeloma line SP2.0. The cells were cultured in 10% fetal calf serum (Hyclone), hypoxanthine and azaserine and wells containing colonies of hybridoma cells were screened for antibody production using antigen-binding ELISA.

Cells from positive wells were cloned at three-tenths cell/well in 10% fetal calf serum (Hyclone), hypoxanthine and positive clones were subcloned one more time in hypoxanthine medium. Capture ELISA (see Example 7) was used for secondary and tertiary screening. This assay offers the advantage that a clone that recognizes the native protein may be selected and thus may be useful for immunoaffinity purification. For example, two monoclonal antibodies (4B11, 8B11) were generated. These antibodies were purified by Gammabind G Sepharose (Pharmacia, Piscataway, NJ) according to manufacturer's procedures and were immobilized to cyanogen bromide - activated Sepharose 4B (Pharmacia, Piscataway, NJ) according to the procedures described by Pharmacia. The ammonium sulphate preparation containing *Cry j I* was applied to the resin and unbound material was washed extensively with PBS. *Cry j I* was eluted with 2 column volumes of 0.1 M glycine, pH 2.7. Silver staining of the eluate fractions run on SDS PAGE showed that *Cry j I* was purified almost to homogeneity. These fractions did not contain detectable levels of *Cry j II*. Other methods to immobilize MAbs 8B11 were also tested. Similar results were obtained using purified MAbs 8B11 covalently cross-linked to Gammabind G Sepharose by dimethylpimelimidate (Schneider C., et al, *J. Biol. Chem.* (1982) volume 257:10766-10769). However, experiments using purified MAbs 8B11 covalently cross-linked to Affi-gel 10 (Biorad, Richmond, CA) showed that although greater than 90% of the monoclonal antibody was covalently coupled to Affi-gel 10, the yield of *Cry j I* purified over this resin was significantly less than that purified from MAbs 8B11 cross-linked to cyanogen bromide-activated Sepharose 4B (data not shown). Nevertheless, the purified *Cry j I* from these monoclonal antibodies immobilized on different resins is still intact and can be recognized by MAbs 8B11 and 4B11 by capture ELISA. Thus, these MAbs will provide a useful tool in purification of *Cry j I* from pollen extracts. Similarly, monoclonal antibodies that bind to recombinant *Cry j I* can also be used for immunoaffinity chromatography. In addition, the monoclonal antibodies generated may be useful for diagnostic purposes. It may also be possible to raise different MAbs that show some specificity towards these different isoforms of *Cry j I* and thus would provide a useful tool to characterize these isoforms.

Example 2

Attempted Extraction of RNA From Japanese Cedar Pollen

Multiple attempts were made to obtain RNA from commercially-available, non-defatted, *Cryptomeria japonica* (Japanese cedar) pollen (Hollister Stier, Seattle, WA). Initially, the method of Sambrook et al., *Molecular Cloning. A Laboratory Manual*, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York (1989) was used in which the sample was suspended and lysed in 4 M guanidine buffer, ground under liquid nitrogen, and pelleted through 5.7 M cesium chloride by ultracentrifugation. Various amounts (3, 5 and 10 g) of pollen in varying amounts of guanidine lysis buffer (10 and 25 ml) were tried. Centrifugation through cesium resulted in viscous material in the bottom of the tube, from which it was not possible to recover an RNA pellet. Although it was possible to obtain RNA from defatted *Ambrosia artemisiifolia* (ragweed) pollen (Greer Laboratories, Lenior, NC) using this protocol, defatting the *Cryptomeria japonica* pollen with acetone before guanidine extraction also did not yield any RNA, as determined by absorbance at A₂₆₀.

An acid phenol extraction of RNA according to the method in Sambrook et al., *supra* was attempted from *Cryptomeria japonica* pollen. The pollen was ground and sheared in 4.5 M guanidine solution, acidified by addition of 2 M sodium acetate, and extracted with water-saturated phenol plus chloroform. After precipitation, the pellet was washed with 4 M lithium chloride, redissolved in 10 mM Tris/5 mM EDTA/1% SDS, chloroform extracted, and re-precipitated with NaCl and absolute ethanol. It was possible to extract *Ambrosia artemisiifolia* but not *Cryptomeria japonica* RNA with this procedure.

Next, 4 g of *Cryptomeria japonica* pollen was suspended in 10 ml extraction buffer (50 mM Tris, pH 9.0, 0.2 M NaCl, 10 mM Mg acetate and diethylpyrocarbonate (DEPC) to 0.1%), ground in a mortar and pestle on dry ice, transferred to a centrifuge tube with 1% SDS, 10 mM EDTA and 0.5% N-lauroyl sarcosine, and the mixture was extracted five times with warm phenol. The aqueous phase was recovered after the final centrifugation, 2.5 vol. absolute ethanol was added, and the mixture was incubated overnight at 4°C. The pellet was recovered by centrifugation, resuspended in 1 ml dH₂O by heating to 65°C, and reprecipitated by

the addition of 0.1 vol. 3 M Na acetate and 2.0 vol. of ethanol. No detectable RNA was recovered in the pellet as judged by absorbance at A₂₆₀ and gel electrophoresis.

Finally, 500 mg of *Cryptomeria japonica* pollen was ground by mortar and pestle on dry ice and suspended in 5 ml of 50 mM Tris pH 9.0 with 0.2 M NaCl, 1 mM EDTA, 1% SDS that had been treated overnight with 0.1% DEPC, as previously described in Frankis and Mascarenhas (1980) *Ann. Bot.* **45**: 595- 599. After five extractions with phenol/chloroform/isoamyl alcohol (mixed at 25:24:1), material was precipitated from the aqueous phase with 0.1 volume 3 M sodium acetate and 2 volumes ethanol. The pellet was recovered by centrifugation, resuspended in dH₂O and heated to 65°C to solubilize the precipitated material.

Further precipitations with lithium chloride were not done. There was no detectable RNA recovered, as determined by absorbance at A₂₆₀ and gel electrophoresis.

In summary, it has not been possible to recover RNA from the commercial pollen. It is not known whether the RNA has been degraded during storage or shipment, or whether the protocols used in this example did not allow recovery of extant RNA. However, RNA was recovered from fresh *Cryptomeria japonica* pollen and staminate cone samples. (See Example 3.)

Example 3

Extraction of RNA From Japanese Cedar Pollen and Staminate Cones and Cloning of *Cry j I*

Fresh pollen and staminate cone samples, collected from a single *Cryptomeria japonica* (Japanese cedar) tree at the Arnold Arboretum (Boston, MA), were frozen immediately on dry ice. RNA was prepared from 500 mg of each sample, essentially as described by Frankis and Mascarenhas, *supra*. The samples were ground by mortar and pestle on dry ice and suspended in 5 ml of 50 mM Tris pH 9.0 with 0.2 M NaCl, 1 mM EDTA, 1% SDS that had been treated overnight with 0.1% DEPC. After five extractions with phenol/chloroform/ isoamyl alcohol (mixed at 25:24:1), the RNA was precipitated from the aqueous phase with 0.1 volume 2 M sodium acetate and 2 volumes ethanol. The pellets were recovered by centrifugation, resuspended in dH₂O and heated to 65°C for 5 min. Two ml of 4 M lithium chloride were added to the RNA preparations and they were incubated

overnight at 0°C. The RNA pellets were recovered by centrifugation, resuspended in 1 ml dH₂O, and again precipitated with 3 M sodium acetate and ethanol overnight. The final pellets were resuspended in 100 µl dH₂O and stored at -80°C.

First strand cDNA was synthesized from 8 µg flowerhead and 4 µg pollen RNA using a commercially available kit (cDNA synthesis systems kit, BRL, Gaithersburg, MD) with oligo dT priming according to the method of Gubler and Hoffman (1983) *Gene* 25: 263-269. An attempt was made to amplify cDNA encoding *Cry j I* using the degenerate oligonucleotide CP-1 (which has the sequence 5'-GATAATCCGATAGATAG-3', wherein T at position 3 can also be C; T at position 6 can also be C; G at position 9 can also be A,T, or C; A at position 12 can also be T, or C; T at position 15 can also be C; A at position 16 can also be T; and G at position 17 can also be C; SEQ ID NO: 3) and primers EDT and ED. Primer EDT has the sequence 5'-GGAATTCTCTAGACTGCAGGTTTTTTTTTT-3'(SEQ ID NO: 24). Primer ED has the sequence 5'-GGAATTCTCTAGACTGCAGGT-3' (SEQ ID NO: 23). CP-1 is the degenerate oligonucleotide sequence encoding the first six amino acids of the amino terminus (AspAsnProIleAspSer, amino acids 1-6 of SEQ ID NO: 1) of *Cry j I*. EDT will hybridize with the poly A tail of the gene. All oligonucleotides were synthesized by Research Genetics, Inc. Huntsville, AL. Polymerase chain reactions (PCR) were carried out using a commercially available kit (GeneAmp DNA Amplification kit, Perkin Elmer Cetus, Norwalk, CT) whereby 10 µl 10x buffer containing dNTPs was mixed with 1 µg of CP- 1 and 1 µg of ED/EDT primers (ED:EDT in a 3:1 M ratio), cDNA (3-5 µl of a 20 µl first strand cDNA reaction mix), 0.5 µl AmpliTaq DNA polymerase, and distilled water to 100 µl.

The samples were amplified with a programmable thermal controller (MJ Research, Inc., Cambridge, MA). The first 5 rounds of amplification consisted of denaturation at 94°C for 1 minute, annealing of primers to the template at 45°C for 1.5 minutes, and chain elongation at 70°C for 2 minutes. The final 20 rounds of amplification consisted of denaturation as above, annealing at 55°C for 1.5 minutes, and elongation as above. Five percent (5 µl) of this initial amplification was then used in a secondary amplification with 1 µg each of CP-2 (which has the sequence 5'- GGGATTCAATTGGGCGCAGAATGG-3' wherein T at position 11 can also be C; G at position 17 can also be A, T, or C; G at position 20 can also be A; T at

position 23 can also be C; and G at position 24 can also be C) (SEQ ID NO: 4), a nested primer, and ED, as above. The sequence 5'-GGGAATTC-3' (bases 1 through 8 of SEQ ID NO: 4) in primer CP-2 represents an *Eco* R1 site added for cloning purposes; the remaining degenerate oligonucleotide sequence encodes amino acids 13-18 of *Cry j I* (AsnTrpAlaGlnAsnArg, amino acids 13 through 18 of SEQ ID NO: 1). Multiple DNA bands were resolved on a 1% GTG agarose gel (FMC, Rockport, ME), none of which hybridized with ³²P end-labeled probe CP-3 (SEQ ID NO: 5) in a Southern blot performed according to the method in Sambrook et al. *supra*. Therefore, it was not possible to select a specific *Cry j I* DNA band and this approach was not pursued. CP-3 has the sequence 5'-
CTGCAGCCATTTCIACATTAAA-3' wherein A at position 9 can also be G; T at position 12 can also be C; A at position 18 can also be G; and A at position 21 can also be G) (SEQ ID NO: 5). Inosine (I) is used at position 15 in place of G or A or T or C to reduce degeneracy (Knoth et al. (1988) *Nucleic Acids Res.* 16: 10932).

The sequence 5'-CTGCAG-3' (bases 1 through 6 of SEQ ID NO: 5) in primer CP-3 represent a Pst I site added for cloning purposes; the remaining degenerate oligonucleotide sequence is the non-coding strand sequence corresponding to coding strand sequence encoding amino acids PheAsnValGluAsnGly (amino acids 327 through 332 of SEQ ID NO: 1) from the internal sequence of *Cry j I*.

A primary PCR was also performed on first-strand cDNA using CP-1 (SEQ ID NO: 3) and CP-3 (SEQ ID NO: 5), as above. A secondary PCR was performed using 5% of the primary reaction using CP-2 (SEQ ID NO: 4) and CP-3 (SEQ ID NO: 5). Again, multiple bands were observed, none of which could be specifically identified in a Southern blot as *Cry j I*, and this approach was also not pursued.

Double-stranded cDNA was then synthesized from approximately 4 µg (pollen) or 8 µg (flowerhead) RNA using a commercially available kit (cDNA Synthesis System kit, BRL, Gaithersburg, MD). After a phenol extraction and ethanol precipitation, the cDNA was blunted with T4 DNA polymerase (Promega, Madison, WI), and ligated to ethanol precipitated, self-annealed, AT (SEQ ID NO: 20) and AL (SEQ ID NO: 22) oligonucleotides for use in a modified Anchored PCR reaction, according to the method in Rafnar et al. (1991) *J. Biol. Chem.* 266: 1229-1236; Frohman et al. (1990) *Proc. Natl. Acad. Sci. USA* 85: 8998-9002; and Roux et

al. (1990) *BioTech.* **8:** 48-57. Oligonucleotide AT has the sequence 5'-GGGTCTAGAGGTACCGTCCGATCGATCATT-3' (SEQ ID NO: 20) (Rafnar et al. *supra*). Oligonucleotide AL has the sequence 5'-AATGATCGATGCT-3' (SEQ ID NO: 22) (Rafnar et al. *Supra*. The amino terminus of *Cry j I* was amplified from the linked cDNA (3 μ l from a 20 μ l reaction) with 1 μ g each of oligonucleotides AP (SEQ ID NO: 21) and degenerate *Cry j I* primer CP-7 (which has the sequence 5'-TTCATICGATTCTGGGCCA-3' wherein G at position 8 can also be T; A at position 9 can also be G; C at position 12 can also be T; and G at position 15 can also be A, T, or C)(SEQ ID NO: 6). Inosine (I) is used at position 6 in place of G or A or T or C to reduce degeneracy (Knoth et al. *supra*). The degenerate oligonucleotide CP-7 (SEQ ID NO: 6) is the non-coding strand sequence corresponding to coding strand sequence encoding amino acids 14-20 (TrpAlaGlnAsnArgMetLys) from the amino terminus of *Cry j I* (amino acids 14-20 of SEQ ID NO: 1). Oligonucleotide AP has the sequence 5'-GGGTCTAGAGGTACCGTCCG-3' (SEQ ID NO: 21).

The primary PCR reaction was carried out as described herein. Five percent (5 μ l) of this initial amplification was then used in a secondary amplification with 1 μ g each of AP (SEQ ID NO: 21) and degenerate *Cry j I* primer CP-8 (SEQ ID NO: 7) an internally nested *Cry j I* oligonucleotide primer, as described herein. Primer CP-8 has the sequence 5'-CCTGCAGCGATTCTGGGCCAAATT-3' wherein G at position 9 can also be T; A at position 10 can also be G; C at position 13 can also be T; G at position 16 can also be A, T, or C; and A at position 23 can also be G)(SEQ ID NO: 7). The nucleotides 5'-CCTGCAG-3' (bases 1 through 7 of SEQ ID NO: 7) represent a *Pst I* restriction site added for cloning purposes. The remaining degenerate oligonucleotide sequence is the non-coding strand sequence corresponding to coding strand sequence encoding amino acids 13-18 of *Cry j I* (AsnTrpAlaGlnAsnArg, amino acids 13-18 of SEQ ID NO: 1) from the amino terminus of *Cry j I*. The dominant amplified product was a DNA band of approximately 193 base pairs, as visualized on an ethidium bromide (EtBr)-stained 3% GTG agarose gel.

Amplified DNA was recovered by sequential chloroform, phenol, and chloroform extractions, followed by precipitation at -20°C with 0.5 volumes of 7.5 ammonium acetate and 1.5 volumes of isopropanol. After precipitation and washing

with 70% ethanol, the DNA was simultaneously digested with *Xba* I and *Pst* I in a 15 μ l reaction and electrophoresed through a preparative 3% GTG NuSieve low melt gel (FMC, Rockport, ME). The appropriate sized DNA band was visualized by EtBr staining, excised, and ligated into appropriately digested M13mp18 for sequencing by the dideoxy chain termination method (Sanger et al. (1977) *Proc. Natl Acad Sci. USA* 74: 5463-5476) using a commercially available sequencing kit (Sequenase kit, U.S. Biochemicals, Cleveland, OH). It was initially thought that ligatable material could only be derived from stamine cone-derived RNA. However, upon subsequent examination, it was shown that ligatable material could be recovered from PCR product generated from pollen-derived RNA, and from stamine cone-derived RNA.

The clone designated JC71.6 was found to contain a partial sequence of *Cry j I*. This was confirmed as an authentic clone of *Cry j I* by having complete identity to the disclosed NH₂-terminal sequence of *Cry j I* (Taniai et al. *supra*). The amino acid at position 7 was determined to be cysteine (Cys) in agreement with the sequence disclosed in U.S. patent 4, 939,239. Amino acid numbering is based on the sequence of the mature protein; amino acid 1 corresponds to the aspartic acid (Asp) disclosed as the NH₂-terminus of *Cry j I* (Taniai et al. *supra*) The initiating methionine was found to be amino acid -21 relative to the first amino acid of the mature protein. The position of the initiating methionine was supported by the presence of upstream in-frame-stop codons and by 78% homology of the surrounding nucleotide sequence with the plant consensus sequence that encompasses the initiating methionine, as reported by Lutcke et al. (1987) *EMBO J.* 6:43-48.

The cDNA encoding the remainder of *Cry j I* gene was cloned from the linker cDNA by using oligonucleotides CP-9 (which has the sequence 5'-ATGGATTCCCCTTGCTTA-3')(SEQ ID NO: 8) and AP (SEQ ID NO: 21) in the primary PCR reaction. Oligonucleotide CP-9 (SEQ ID NO: 8) encodes amino acids MetAspSerProCysLeu of *Cry j I* (amino acids -21 through -16 of SEQ ID NO: 1) from the leader sequence of *Cry j I*, and is based on the nucleotide sequence determined for the partial *Cry j I* clone JC76.1.

A secondary PCR reaction was performed on 5% of the initial amplification mixture, with 1 μ g each of AP (SEQ ID NO: 21) and CP-10 (which

has the sequence 5'-GGGAATTCGATAATCCCATAGACAGC-3')(SEQ ID NO: 9),
the nested primer. The nucleotide sequence 5'-GGGAATT-3' of primer CP- 10
(bases 1 through 8 of SEQ ID NO: 9) represent an *Eco* RI restriction site added for
cloning purposes. The remaining oligonucleotide sequence encodes amino acids 1-6
5 of *Cry j I* (AspAsnProIleAspSer) (amino acids 1 through 6 of SEQ ID NO: 1), and is
based on the nucleotide sequence determined for the partial *Cry j I* clone JC76.1.
The amplified DNA product was purified and precipitated as above, followed by
digestion with *Eco* RI and *Xba* I and electrophoresis through a preparative 1% low
melt gel. The dominant DNA band was excised and ligated into M13mp19 and
10 pUC19 for sequencing. Again, ligatable material was recovered from cDNA
generated from pollen-derived RNA, and from stamine cone-derived RNA. Two
clones, designated pUC19JC91a and pUC19JC91d, were selected for full-length
sequencing. They were subsequently found to have identical sequences.

DNA was sequenced by the dideoxy chain termination method
15 (Sanger et al. *supra*) using a commercially available kit (sequenase kit (U.S.
Biochemicals, Cleveland, OH). Both strands were completely sequenced using M13
forward and reverse primers (N.E. Biolabs, Beverly, MA) and internal sequencing
primers CP-13 (SEQ ID NO: 10), CP-14 (SEQ ID NO: 11), CP-15 (SEQ ID NO:
12), CP-16 (SEQ ID NO: 13), CP-18 (SEQ ID NO: 15), CP-19 (SEQ ID NO: 16),
20 and CP-20 (SEQ ID NO: 17). CP-13 has the sequence 5'-
ATGCCTATGTACATTGC-3' (SEQ ID NO: 10). CP-13 (SEQ ID NO: 10) encodes
amino acids 82-87 of *Cry j I* (MetProMetTyrIleAla, amino acids 82 through 87 of
SEQ ID NO: 1). CP-14 has the sequence 5'-GCAATGTACATAGGCAT-3' (SEQ
ID NO: 11) and corresponds to the non-coding strand sequence of CP-13 SEQ ID
25 NO: 10). CP-15 has the sequence 5'- TCCAATTCTTCTGATGGT-3' ((SEQ ID
NO: 12) which encodes amino acids 169-174 of *Cry j I* (SerAsnSerSerAspGly,
amino acids 169 through 174 of SEQ ID NO: 1). CP-16 has the sequence 5'-
TTTGTCAATTGAGGAGT-3' (SEQ ID NO: 13) which is the non-coding strand
sequence which corresponds to coding strand sequence encoding amino acids 335-
30 340 of *Cry j I* (ThrProGlnLeuThrLys, amino acids 335 through 340 of SEQ ID NO:
1). CP-18 has the sequence 5'-TAGCAAATCCAGTCGAAGT-3' (SEQ ID NO: 15)
which is the non-coding strand sequence which substantially corresponds to coding
strand sequence encoding amino acids 181 through 186 of *Cry j I*

(ThrSerThrGlyValThr, amino acids 181 through 186 of SEQ ID NO: 1) except that the fourth nucleotide of CP-18 (SEQ ID NO: 15) was synthesized as a C rather than the correct nucleotide, T. CP-19 which has the sequence 5'-
5 TAGCTCTCATTTGGTGC-3' (SEQ ID NO: 16) is the non-coding strand sequence which corresponds to coding strand sequence encoding amino acids 270 through 275 of *Cry j I* (AlaProAsnGluSerTyr, amino acids 270 through 275 of SEQ ID NO: 1). CP-20 has the sequence 5'- TATGCAATTGGTGGGAGT-3' (SEQ ID NO: 17)
which is the coding strand sequence for amino acids 251-256 of *Cry j I*
10 (TyrAlaIleGlyGlySer, amino acids 251 through 256 of SEQ ID NO: 1). The sequenced DNA was found to have the sequence shown in Figs. 4a and 4b (SEQ ID NO: 1). This is a composite sequence from the two overlapping clones JC 71.6 and pUC19J91a. The complete cDNA sequence for *Cry j I* is composed of 1312 nucleotides, including 66 nucleotides of 5' untranslated sequence, an open reading frame starting with the codon for an initiating methionine, of 1122 nucleotides, and a 15 3' untranslated region. There is a consensus polyadenylation signal sequence in the 3' untranslated region 25 nucleotides 5' to the poly A tail (nucleotides 1279-1283 of Fig 4 and SEQ. ID NO: 1). Nucleotides 1313-1337 of Fig. 4 and SEQ. ID NO: 1 represent vector sequences. The position of the initiating methionine is confirmed by the presence of in-frame upstream stop codons and by 78% homology with the 20 plant consensus sequence that encompasses the initiating methionine
(AAAAAUGGGA (bases 62 through 70 of SEQ ID NO: 1) found in *Cry j I* compared with the AACAAUGGC consensus sequence for plants, Lutcke et al. (1987) *EMBO J.* **6:** 43-48). The open reading frame encodes a protein of 374 amino acids of which the first 21 amino acids comprise a leader sequence that is cleaved from the mature 25 protein. The amino terminus of the mature protein was identified by comparison with the published NH₂-terminal sequence (Taniai et al. (1988) *supra*) and with sequence determined by direct amino acid analysis of purified native *Cry j I*
(Example 1). The deduced amino acid sequence of the mature protein, comprised of 30 353 amino acids has complete sequence identity with the published protein sequence for *Cry j I* (Taniai et al. *supra*), including the first twenty amino acids for the NH₂- terminal and sixteen contiguous internal amino acids. The mature protein also contains five potential N-linked glycosylation sites corresponding to the consensus sequence N-X-S/T.

Example 4

Extraction of RNA from Japanese Cedar Pollen Collected in Japan

5 Fresh pollen collected from a pool of *Cryptomeria japonica* (Japanese cedar) trees in Japan was frozen immediately on dry ice. RNA was prepared from 500 mg of the pollen, essentially as described by Frankis and Mascarenhas *Ann. Bot.* 45:595-599. The samples were ground by mortar and pestle on dry ice and
10 suspended in 5 ml of 50 mM Tris pH 9.0 with 0.2 M NaCl, 1 mM EDTA, 1% SDS that had been treated overnight with 0.1% DEPC. After five extractions with phenol/chloroform/isoamyl alcohol (mixed at 25:24:1), the RNA was precipitated from the aqueous phase with 0.1 volume 3 M sodium acetate and 2 volumes ethanol. The pellets were recovered by centrifugation, resuspended in dH₂O and heated to
15 65°C for 5 minutes. Two ml of 4 M lithium chloride were added to the RNA preparations and they were incubated overnight at 9°C. The RNA pellets were recovered by centrifugation, resuspended in 1 ml dH₂O, and again precipitated with 3 M sodium acetate and ethanol overnight. The final pellets were resuspended in 100 µl dH₂O and stored at -80°C.

20 Double stranded cDNA was synthesized from 8 µg pollen RNA using the cDNA Synthesis Systems kit (BRL) with oligo dT priming according to the method of Gubler and Hoffman (1983) *Gene* 25:263-269. Polymerase chain reactions (PCR) were carried out using the GeneAmp DNA Amplification kit (Perkin Elmer Cetus) whereby 10 µl 10x buffer containing dNTPs was mixed with
25 100 pmol each of a sense oligonucleotide and an anti-sense oligonucleotide, (10 µl of a 400 µl double stranded cDNA reaction mix), 0.5 µl AmpliTaq DNA polymerase, and distilled water to 100 µl.

30 The samples were amplified with a programmable thermal controller from MJ Research, Inc. (Cambridge, MA). The first 5 rounds of amplification consisted of denaturation at 94°C for 1 minute, annealing of primers to the template at 45°C for 1 minute, and chain elongation at 72°C for 1 minute. The final 20 rounds of amplification consisted of denaturation as above, annealing at 55°C for 1 minute, and elongation as above.

Seven different *Cry j I* primer pairs were used to amplify the double stranded cDNA as follows: CP-9 (SEQ. ID #8) and CP-17 (SEQ. ID #14), CP-10 (SEQ. ID #9) and CP-17 (SEQ. ID #14), CP-10 (SEQ. ID #9) and CP-16 (SEQ. ID #13), CP-10 (SEQ. ID #9) and CP-19 (SEQ. ID #16), CP-10 (SEQ. ID #9) and CP-18 (SEQ. ID #15), CP-13 (SEQ. ID #10) and CP-17 (SEQ. ID #14), and CP-13 (SEQ. ID #10) and CP-19 (SEQ. ID #16). CP-17 (SEQ. ID #14) has the sequence 5'-
5 CCTGCAGAACGCTTCATCAACAAACGTTAGA-3' and corresponds to non-coding strand sequence that corresponds to coding strand sequence encoding amino acids SKRC* (amino acids 350-353 and the stop codon of SEQ. ID #1). The nucleotide sequence 5'-CCTGCAGAACGCTT-3' (bases 1 through 13 of SEQ. ID #14)
10 represents *Pst* I and *Hin* dIII restriction sites added for cloning purposes. The nucleotide sequence 5'-TCA-3' (bases 13 through 15 of SEQ. ID #14) correspond to the non-coding strand sequence of a stop codon. All of the amplifications yielded products of the expected size when viewed on ethidium bromide (EtBr)-stained
15 agarose gels. Two of these primer pairs were used in amplifications whose products were cloned into pUC19 for full-length sequencing. The PCR reaction with CP-10 (SEQ. ID #9) and CP-16 (SEQ. ID #13) on the double stranded cDNA yielded a band of approximately 1.1 kb, and was called JC130. A separate first strand cDNA reaction was done with 8 µg pollen RNA as described above and amplified with
20 oligonucleotide primers CP-10 (SEQ. ID #9) and CP-17 (SEQ. ID #14). This amplification yielded a full-length cDNA, named JC135, from the amino terminus of the mature protein to the stop codon.

Amplified DNA was recovered by sequential chloroform, phenol, and chloroform extractions, followed by precipitation at -20°C with 0.5 volumes of 7.5
25 ammonium acetate and 1.5 volumes of isopropanol. After precipitation and washing with 70% ethanol, the DNA was blunted with T4 polymerase followed by digestion with *Eco* RI, in the case of JC130, or simultaneously digested with *Eco* RI and *Pst* I, in the case of JC135, in a 15 µl reaction and electrophoresed through a preparative 1% SeaPlaque low melt gel (FMC). Appropriate sized DNA bands were visualized by EtBr staining, excised, and ligated into appropriately digested pUC19 for dideoxy DNA sequencing by the dideoxy chain termination method (Sanger et al. (1977) *Proc. Natl. Acad. Sci. USA* 74:5463-5476) using a commercially available sequencing kit (Sequenase kit, U.S. Biochemicals, Cleveland, OH).

Both strands were sequenced using M13 forward and reverse primers (N.E. Biolabs, Beverly, MA) and internal sequencing primers CP-13 (SEQ. ID #10), CP-15 (SEQ. ID #12), CP-16 (SEQ ID #13), CP-18 (SEQ. ID #15), CP-19 (SEQ. ID #16) and CP-20 (SEQ. ID #17). Two clones from amplification JC130 (JC130a and JC130b) and one clone from amplification JC135 (JC135g) were found to be *Cry j I* clones upon sequencing. The nucleotide and deduced amino acid sequences of clones JC130a and JC135g were identical to previously known *Cry j I* sequence (SEQ. ID #1). Clone JC130b was found to contain a single nucleotide difference from the previously known *Cry j I* sequence (SEQ. ID #1). Clone JC130b had a C at nucleotide position 306 of Seq. ID #1. This nucleotide change results in a predicted amino acid change from a Tyr to a His at amino acid 60 of the mature *Cry j I* protein. This polymorphism has not yet been confirmed in an independently-derived PCR clone or by direct amino acid sequencing. However, such polymorphisms in primary nucleotide and amino acid sequences are expected.

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Example 5

Expression of *Cry j I*

Expression of *Cry j I* was performed as follows. Ten µg of pUC19JC91a was digested with *Xba* I, precipitated, then blunted with T4 polymerase. *Bam* HI linkers (N.E. Biolabs, Beverly, MA) were blunt-end ligated to pUC19JC91a overnight and excess linkers were removed by filtration through a NACS ion exchange minicolumn (BRL, Gaithersburg, MD). The linkerd cDNA was then digested simultaneously with *Eco* RI and *Bam* HI. The *Cry j I* insert (extending from the nucleotides encoding the amino terminus of the mature protein through the stop codon) was isolated by electrophoresis of this digest through a 1% SeaPlaque low melt agarose gel. The insert was then ligated into the appropriately digested expression vector pET-11d (Novagen, Madison, WI; Jameel et al. (1990) *J. Virol.* 64:3963-3966) modified to contain a sequence encoding 6 histidines (His 6) immediately 3' of the ATG initiation codon followed by a unique *Eco* RI endonuclease restriction site. A second *Eco* RI endonuclease restriction site in the vector, along with neighboring *Cla* I and *Hind* III endonuclease restriction sites, had previously been removed by digestion with *Eco* RI and *Hind* III, blunting and religation. The histidine (His6) sequence was added for affinity purification of the

recombinant protein (*Cry j I*) on a Ni²⁺ chelating column (Hochuli et al. (1987) *J. Chromatog.* 411:177-184; Hochuli et al. (1988) *Bio/Tech.* 6:1321-1325.). A recombinant clone was used to transform *Escherichia coli* strain BL21-DE3 which harbors a plasmid that has an isopropyl-β-D-thiogalactopyranoside (IPTG)-inducible promoter preceding the gene encoding T7 polymerase. Induction with IPTG leads to high levels of T7 polymerase expression, which is necessary for expression of the recombinant protein in pET-11d, which has a T7 promoter. Clone pET-11dΔHRhis6JC91a.d was confirmed by dideoxy sequencing (Sanger et al. *Supra*) with CP-14 (SEQ. ID #11) to be a *Cry j I* clone in the correct reading frame for expression.

Expression of the recombinant protein was confirmed in an initial small culture (50 ml). An overnight culture of clone pET-11dΔHRhis6JC91a.d was used to inoculate 50 ml of media (Brain Heart Infusion Media, Difco) containing ampicillin (200 µg/ml), grown to an A₆₀₀ = 1.0 and then induced with IPTG (1 mM, final concentration) for 2 hrs. One ml aliquots of the bacteria were collected before and after induction, pelleted by centrifugation, and crude cell lysates prepared by boiling the pellets for 5 minutes in 50 mM Tris HCl, pH 6.8, 2 mM EDTA, 1% SDS, 1% β-mercaptoethanol, 10% glycerol, 0.25% bromophenol blue (Studier et al., (1990) *Methods in Enzymology* 185:60-89). Recombinant protein expression was visualized as a band with the predicted molecular weight of approximately 38 kDa on a Coomassie blue-stained SDS-PAGE gel, according to the method in Sambrook et al., *supra*, on which 40 µl of the crude lysate was loaded. A negative control consisted of crude lysates from uninduced bacteria containing the plasmid with *Cry j I* and an induced lysate from bacteria carrying no plasmid.

The pET-11dΔ HRhis6JC91a.d clone was then grown on a large scale for recombinant protein expression and purification. A 2 ml culture bacteria containing the recombinant plasmid was grown for 8 hr, then streaked onto solid media (e.g. 6 petri plates (100 x 15 mm) with 1.5% agarose in LB medium (Gibco-BRL, Gaithersburg, MD) containing 200 µg/ml ampicillin), grown to confluence overnight, then scraped into 9 L of liquid media (Brain Heart Infusion media, Difco) containing ampicillin (200 µg/ml). The culture was grown until the A₆₀₀ was 1.0, IPTG added (1 mM final concentration), and the culture grown for an additional 2 hours.

Bacteria were recovered by centrifugation (7,930 x g, 10 min), and lysed in 90 ml of 6M Guanidine-HCl, 0.1M Na₂HPO₄, pH 8.0 for 1 hour with vigorous shaking. Insoluble material was removed by centrifugation (11,000 x g, 10 min, 4° C). The pH of the lysate was adjusted to pH 8.0, and the lysate applied to an 80 ml Nickel NTA agarose column (Qiagen) that had been equilibrated with 6 M Guanidine HCl, 100 mM Na₂HPO₄, pH 8.0. The column was sequentially washed with 6 M Guanidine HCl, 100 mM Na₂HPO₄, 10 mM Tris-HCl, pH 8.0, then 8 M urea, 100 mM Na₂HPO₄, pH 8.0, and finally 8 M urea, 100 mM sodium acetate, 10 mM Tris-HCl, pH 6.3. The column was washed with each buffer until the flow through had an A₂₈₀ ≤ 0.05.

The recombinant protein, *Cry j I*, was eluted with 8 M urea, 100 mM sodium acetate, 10 mM Tris-HCl, pH 4.5, and collected in 10 ml aliquots. The protein concentration of each fraction was determined by absorbance at A₂₈₀ and the peak fractions pooled. An aliquot of the collected recombinant protein was analyzed on SDS-PAGE according to the method in Sambrook et al., *supra*.

The first 9 L prep, JCpET-1, yielded 30 mg of *Cry j I* with approximately 78% purity, as determined by densitometry (Shimadzu Flying Spot Scanner, Shimadzu Scientific Instruments, Inc., Braintree, MA) of the Coomassie-blue stained SDS-PAGE gel. A second 9 L prep prepared the same way, JCpET-2, yielded 41 mg of *Cry j I* with approximately 77% purity.

Example 6

Japanese Cedar Pollen Allergic Patient T Cell Studies with *Cry j I* - the Primary Cedar Pollen Antigen.

Synthesis of Overlapping Peptides

Japanese cedar pollen *Cry j I* overlapping peptides were synthesized using standard Fmoc/tBoc synthetic chemistry and purified by Reverse Phase HPLC. Figure 13 shows *Cry j I* peptides used in these studies. The peptide names are consistent throughout.

T Cell Responses to Cedar Pollen Antigen Peptides

Peripheral blood mononuclear cells (PBMC) were purified by lymphocyte separation medium (LSM) centrifugation of 60 ml of heparinized blood from Japanese cedar pollen-allergic patients who exhibited clinical symptoms of seasonal rhinitis and were MAST and/or skin test positive for Japanese cedar pollen. Long term T cell lines were established by stimulation of 2×10^6 PBL/ml in bulk cultures of complete medium (RPMI-1640, 2 mM L-glutamine, 100 U/ml penicillin/streptomycin, 5×10^{-5} M 2-mercaptoethanol, and 10 mM HEPES supplemented with 5% heat inactivated human AB serum) with 20 μ g/ml of partially purified native *Cry j I* (75% purity containing three bands similar to the three bands in Fig. 2) for 7 days at 37°C in a humidified 5% CO₂ incubator to select for *Cry j I* reactive T cells. This amount of priming antigen was determined to be optimal for the activation of T cells from most cedar pollen allergic patients. Viable cells were purified by LSM centrifugation and cultured in complete medium supplemented with 5 units recombinant human IL-2/ml and 5 units recombinant human IL-4/ml for up to three weeks until the cells no longer responded to lymphokines and were considered "rested". The ability of the T cells to proliferate to selected peptides, recombinant *Cry j I* (*rCry j I*), purified native *Cry j I*, or recombinant *Amb a I.1* (*rAmb aI.1*) was then assessed. For assay, 2×10^4 rested cells were restimulated in the presence of 2×10^4 autologous Epstein-Barr virus (EBV)-transformed B cells (prepared as described below) (gamma-irradiated with 25,000 RADS) with 2-50 μ g/ml of *rCry j I*, purified native *Cry j I* or *rAmb a I.1*, in a volume of 200 μ l complete medium in duplicate or triplicate wells in 96-well round bottom plates for 2-4 days. The optimal incubation was found to be 3 days. Each well then received 1 μ Ci tritiated thymidine for 16-20 hours. The counts incorporated were collected onto glass fiber filter mats and processed for liquid scintillation counting. Fig. 12 shows the effect of varying antigen dose in assays with recombinant *Cry j I*, purified native *Cry j I*, and recombinant *Amb a I.1* and several antigenic peptides synthesized as described above. Some peptides were found to be inhibitory at high concentrations in these assays. The titrations were used to optimize the dose of peptides in T cell assays. The maximum response in a titration of each peptide is expressed as the stimulation index (S.I.). The S.I. is the counts per minute (CPM) incorporated by cells in response to peptide, divided by the CPM incorporated by cells in medium only. An S.I. value equal to or greater than 2 times the background level is considered

"positive" and indicates that the peptide contains a T cell epitope. The positive results were used in calculating mean stimulation indices for each peptide for the group of patients tested. The results shown in Fig. 12 demonstrate that patient #999 responds well to recombinant *Cry j I*, and purified native *Cry j I*, as well as to peptides CJ1-2, 3, 20, and 22 but not to recombinant *Amb a I.1*. This indicates that *Cry j I* T cell epitopes are recognized by T cells from this particular allergic patient and that r*Cry j I* and peptides CJ1-2, 3, 20 and 22 contain such T cell epitopes. Furthermore, the epitopes were often not detected with the adjacent overlapping peptides, and therefore probably span the non-overlapping central residues of the reactive peptides. No significant crossreactivity was found in T cell assays using T cells primed with control antigens or with *Cry j I* primed T cells against other antigens.

The above procedure was followed with a number of other patients. Individual patient results were used in calculating the mean S.I. for each peptide if the patient responded to the *Cry j I* protein at an S.I. of 2.0 or greater and the patient responded to at least one peptide derived from *Cry j I* at an S.I. of 2.0 or greater. A summary of positive experiments from seventeen patients is shown in Figure 14. The bars represent the positivity index. Above each bar is the percent of positive responses with an S.I. of at least two to the peptide or protein in the group of patients tested. In parenthesis above each bar are the mean stimulation indices for each peptide or protein for the group of patients tested. All seventeen T cell lines responded to purified native *Cry j I* and r*Cry j I*. These seventeen T cell lines also responded at a significantly lower level to r*Amb a I.1* indicating that the *Amb a I* allergens share a degree of homology with *Cry j I* and that "shared" T cell epitopes might exist between *Cry j I* and *Amb a I*. This panel of Japanese cedar allergic patients responded to peptides CJ1-1, CJ1-2, CJ1-3, CJ1-4, CJ1-7, CJ1-8, CJ1-9, CJ1-10, CJ1-11, CJ1-12, CJ1-14, CJ1-15, CJ1-16, CJ1-17, CJ1-18, CJ1-19, CJ1-20, CJ1-21, CJ1-22, CJ1-23, CJ1-24, CJ1-25, CJ1-26, CJ1-27, CJ1-28, CJ1-30, CJ1-31, CJ1-32, CJ1-33, CJ1-34 and CJ1-35 indicating that these peptides contain T cell epitopes.

Preparation of (EBV)-transformed B Cells for Use as Antigen Presenting Cells

Autologous EBV-transformed cell lines were γ -irradiated with 25,000 Rad and used as antigen presenting cells in secondary proliferation assays and secondary bulk stimulations. These cell lines were also used as a control in the immuno-fluorescence flow cytometry analysis. These EBV-transformed cell lines were made by incubating 5×10^6 PBL with 1 ml of B-59/8 Marmoset cell line (ATCC CRL1612, American Type Culture Collection, Rockville, MD) conditioned medium in the presence of 1 μ g/ml phorbol 12-myristate 13-acetate (PMA) at 37°C for 60 minutes in 12 X 75 mm polypropylene round-bottom Falcon snap cap tubes (Becton Dickinson LABware, Lincoln Park, NJ). These cells were then diluted to 1.25×10^6 cells/ml in RPMI-1640 as described above except supplemented with 10% heat-inactivated fetal bovine serum and cultured in 200 μ l aliquots in flat bottom culture plates until visible colonies were detected. They were then transferred to larger wells until the cell lines were established.

Example 7

20 *Cry j I* as the Major Cedar Pollen Allergen

To examine the importance of *Cry j I*, reported as the major allergen of Japanese cedar pollen, both direct and competition ELISA assays were performed. For the direct ELISA assays, wells were coated with either soluble pollen extract (SPE) of Japanese cedar pollen or purified native *Cry j I* (assayed at 90% purity by protein sequencing) and human IgE antibody binding to these antigens was analyzed. Pooled human plasma, consisting of an equal volume of plasma from 15 patients with a Japanese cedar pollen MAST score of 2.5 or greater, and two individual patient plasma samples were compared in this assay. Fig. 5 shows the results of the binding reactivity with these two antigens. The overall pattern of binding is very similar whether the coating antigen is SPE (Fig. 5a) or purified native *Cry j I* (Fig. 5b).

In the competition assay, ELISA wells were coated with Japanese cedar

pollen SPE and then allergic patient IgE binding was measured in the presence of competing purified native *Cry j I* in solution. The source of allergic IgE in these assays was either the pool of plasma from 15 patients (denoted PHP) or seven individual plasma samples from patients with a Japanese cedar MAST score of 2.5 or greater. The competition assay using the pooled human plasma samples compares the competitive binding capacity of purified native *Cry j I* to Japanese cedar pollen SPE and an irrelevant allergen source, rye grass SPE. Fig. 6 shows the graphed results of the competition ELISA with pooled human plasma. The concentration of protein present in the Japanese cedar pollen SPE is approximately 170 times greater at each competing point than is the purified native *Cry j I*. From this analysis it is clear that the purified native *Cry j I* competes very well for IgE binding to the whole range of proteins present in the Japanese cedar pollen soluble pollen extract. This implies that most of the anti-*Cry j I* IgE reactivity is directed against native *Cry j I*. The negative control shows no specific competitive activity and the competing SPE in solution can completely remove binding to the coated wells. This assay was repeated with individual patients as a measure of the range of the IgE response within the allergic population. Fig. 7 shows this result where the competition of binding to SPE was performed with purified native *Cry j I*. The results demonstrate that although the patients show different dose response to Japanese cedar pollen SPE, each of the seven patients' IgE binding to Japanese cedar pollen SPE could be competed with purified native *Cry j I*. The implications of these data are that for each patient the IgE reactivity directed against *Cry j I* is predominant but that there is variation in this reactivity between patients. The overall conclusion is that these data support the previous findings (Yasueda et al., (1988) *supra*) that *Cry j I* is the major allergen of Japanese cedar pollen.

The reactivity of IgE from cedar pollen allergic patients to the pollen proteins is dramatically reduced when these proteins are denatured. One method of analyzing this property is through direct binding ELISA where the coating antigen is the Japanese cedar pollen SPE or denatured Japanese cedar pollen SPE which has been denatured by boiling in the presence of a reducing agent DTT. This is then examined with allergic patient plasma for IgE binding reactivity. Fig. 8a, shows the direct binding assay to the SPE with seven individual plasma samples. In Fig. 8b, the binding results with the denatured SPE demonstrates the marked decrease in

reactivity following this treatment. To determine the extent of *Cry j I* binding to the ELISA wells, *Cry j I* was detected with a rabbit polyclonal antisera against the *Amb a I & II* protein family. These ragweed proteins have high sequence identity (46%) with *Cry j I* and this antisera can be used as a cross reactive antibody detection system. In conclusion, these data demonstrate a marked loss in IgE reactivity following denaturation of the Japanese cedar pollen SPE.

5 **Example 8**

10 **IgE Reactivity and Histamine Release Analysis**

15 The recombinant *Cry j I* protein (*rCry j I*), expressed in bacteria and then purified (as described in Example 5), has been examined for IgE reactivity. The first method applied to this examination was direct ELISA where wells were coated with the recombinant *Cry j I* and IgE binding was assayed on individual patients. Fig. 9 is the graphic representation of this direct ELISA. The only positive signals on this data set are from the two control antisera rabbit polyclonal anti-*Amb a I & II* prepared by conventional means (Rabbit anti-*Amb a I & II*) and CBF2, a monoclonal antibody raised against *Amb a I* that cross reacts with *Cry j I*. By this method all patients tested showed no IgE reactivity with the recombinant *Cry j I*.

20 Another method of analysis that was applied to the examination of IgE reactivity to the recombinant *Cry j I* was a capture ELISA. This analysis relies on the use of a defined antibody, in this case CBF2 to bind the antigen and allow for the binding of antibodies to other epitope sites. The format of this capture ELISA is 1) wells are coated with MAb CBF2, 2) antigen or PBS (as one type of negative control) is added and captured by specific interaction with the coated MAb, 3) either the control antibody anti-*Amb a I & II* (Fig. 10b) or human allergic plasma (Fig. 10a) is added as the detecting antibody, and 4) detection of antibody binding is assayed. Figs. 10a and 10b are the graphed results of these assays. For the IgE analysis, the pooled human plasma (PHP) (15 patients) was used. The conclusion from these results is that there is no indication of any specific binding of human allergic IgE to *rCry j I* by this method of analysis. However, the capture of *rCry j I* works as evidenced by the control antibody binding curve, shown in Fig. 10b. The lack of IgE binding to *E. coli* expressed *rCry j I* may be due to absence of

carbohydrate or any other post-translational modification and/or that the majority of IgE cannot react with denatured *Cry j I*. RAST, competition ELISA and Western blotting data also demonstrates no specific IgE reactivity to the r*Cry j I* (data not shown).

5 A histamine release assay was performed on one Japanese cedar pollen allergic patient using Japanese cedar pollen SPE, purified native *Cry j I* and r*Cry j I* as the added antigens. This assay is a measure of IgE reactivity through human basophil mediator release. The results of this assay, shown in Fig. 11, demonstrate strong histamine release with both purified native *Cry j I* and the Japanese cedar pollen SPE over a wide concentration range. The only point where there is any measurable histamine release with the *Cry j I* is at the highest concentration, 50 µg/ml. Two possible explanations for this release by the r*Cry j I* are: 1) specific reactivity with a very low proportion of the anti-*Cry j I* IgE capable of recognizing the recombinant form of *Cry j I*, or 2) non-specific release caused by low abundance of bacterial contaminants observed only at the highest antigen concentration. Thus far, this result has only been shown in a single patient. In addition, the data shown are from single data points at each protein concentration.

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It may be possible to use this recombinantly expressed *Cry j I* protein for immunotherapy as *E. coli* expressed material has T cell reactivity (Example 6), but does not appear to bind IgE from *Cryptomeria japonica* atopes nor cause histamine release from the mast cells and basophils of such atopes *in vitro*. Expression of r*Cry j I* which is capable of binding IgE could possibly be achieved in yeast, insect (baculovirus) or mammalian cells (e.g. CHO, human and mouse). A r*Cry j I* capable of actively binding IgE may be important for the use of recombinant material for diagnostic purposes.

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To analyze IgE reactivity to selected *Cry j I* peptides a direct ELISA format was used. ELSIA wells were coated with 25 peptides derived from *Cry j I* and assayed for IgE binding. Fig. 15a and 15b are graphs of these binding results using PHP (15 patients) as the cedar pollen allergic IgE source. This pool of plasma was formulated for enrichment of IgE that could bind to denatured SPE (as determined by direct ELISA) and therefore increase the chance of reactivity toward the peptides. In this assay, the peptide IgE binding capacity was compared to that of purified native *Cry j I* and to r*Cry j I*. The only specific IgE detected in this assay was to

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purified native *Cry j I* which supports the finding that Japanese cedar allergic patient IgE does not bind to recombinant *Cry j I* or the recombinant *Cry j I* peptides tested (Fig. 15).

5 Although the invention has been described with reference to its preferred embodiments, other embodiments, can achieve the same results. Variations and modifications to the present invention will be obvious to those skilled in the art and it is intended to cover in the appended claims all such modification and equivalents and follow in the true spirit and scope of this invention.

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